

(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization  
International Bureau



(43) International Publication Date  
4 October 2001 (04.10.2001)

PCT

(10) International Publication Number  
**WO 01/73052 A2**

(51) International Patent Classification<sup>7</sup>: **C12N 15/54**,  
15/61, 15/31, 15/70, 9/90, 9/12, 1/21, C07K 14/195, 16/40

(21) International Application Number: PCT/US01/09950

(22) International Filing Date: 28 March 2001 (28.03.2001)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:  
60/192,736 28 March 2000 (28.03.2000) US

(81) Designated States (*national*): AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DZ, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, TZ, UA, UG, UZ, VN, YU, ZA, ZW.

(84) Designated States (*regional*): ARIPO patent (GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE, TR), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).

(71) Applicant and

(72) Inventor: **MCHENRY, Charles, S.** [US/US]; 191 University Boulevard #305, Denver, CO 80206 (US).

Published:

— without international search report and to be republished upon receipt of that report

(74) Agents: **GOLDSTEIN, Jorge, A.** et al.; Sterne, Kessler, Goldstein & Fox P.L.L.C., Suite 600, 1100 New York Avenue, N.W., Washington, DC 20005-3934 (US).

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

(54) Title: NOVEL THERMOPHILIC POLYMERASE III HOLOENZYME

(57) Abstract: The present invention relates to gene and amino acid sequences encoding DNA polymerase III holoenzyme subunits and structural genes from thermophilic organisms. In particular, the present invention provides DNA polymerase III holoenzyme subunits and accessory proteins of *T. thermophilus*. The present invention also provides antibodies, primers, probes, and other reagents useful to identify DNA polymerase III molecules.



WO 01/73052 A2

NOVEL THERMOPHILIC POLYMERASE III HOLOENZYME  
BACKGROUND OF THE INVENTION

Field of the Invention

5

The present invention relates to gene and amino acid sequences encoding DNA polymerase III holoenzyme subunits and structural genes from thermophilic organisms. In particular, the present invention provides DNA polymerase III holoenzyme subunits and accessory proteins of *T.*  
10 *thermophilus*. The present invention also provides antibodies and other reagents useful to identify DNA Polymerase III molecules.

Background Art

15

Bacterial cells contain three types of DNA polymerases termed polymerase I, II and III. DNA polymerase III (pol III) is responsible for the replication of the majority of the chromosome. Pol III is referred to as a replicative polymerase; replicative polymerases are rapid and highly processive enzymes. Pol I and II are referred to as non-replicative  
20 polymerases although both enzymes appear to have roles in replication. DNA polymerase I is the most abundant polymerase and is responsible for some types of DNA repair, including a repair-like reaction that permits the joining of Okazaki fragments during DNA replication. Pol I is essential for the repair of DNA damage induced by UV irradiation and radiomimetic drugs. Pol II is  
25 thought to play a role in repairing DNA damage which induces the SOS response and in mutants which lack both pol I and III, pol II repairs UV-induced lesions. Pol I and II are monomeric polymerases while pol III comprises a multisubunit complex.

25

30

In *E. coli*, pol III comprises the catalytic core of the *E. coli* replicase. In *E. coli*, there are approximately 400 copies of DNA polymerase I per cell, but only 10-20 copies of pol III (Kornberg and Baker, *DNA Replication*, 2d ed., W.H. Freeman & Company, [1992], pp. 167; and Wu *et al.* J. Biol. Chem., 259:12117-12122 [1984]). The low abundance of pol III and its relatively feeble activity on gapped DNA templates typically used as a general

- 2 -

replication assays delayed its discovery until the availability of mutants defective in DNA polymerase I (Kornberg and Gefter, J. Biol. Chem., 47:5369-5375 [1972]).

The catalytic subunit of pol III is distinguished as a component of *E. coli* major replicative complex, apparently not by its intrinsic catalytic activity, but by its ability to interact with other replication proteins at the fork. These interactions confer upon the enzyme enormous processivity. Once the DNA polymerase III holoenzyme associates with primed DNA, it does not dissociate for over 40 minutes—the time required for the synthesis of the entire 4 Mb *E. coli* chromosome (McHenry, Ann. Rev. Biochem., 57:519-550 [1988]). Studies in coupled rolling circle models of the replication fork suggest the enzyme can synthesize DNA 150 kb or longer without dissociation *in vitro* (Mok and Marians, J. Biol. Chem., 262:16644-16654 [1987]; Wu *et al.*, J. Biol. Chem., 267:4030-4044 [1992]). The essential interaction required for this high processivity is an interaction between the  $\alpha$  catalytic subunit and a dimer of  $\beta$ , a sliding clamp processivity factor that encircles the DNA template like a bracelet, permitting it to rapidly slide along with the associated polymerase, but preventing it from falling off (LaDuca *et al.*, J. Biol. Chem., 261:7550-7557 [1986]; Kong *et al.*, Cell 69:425-437 [1992]). The  $\beta$ - $\alpha$  association apparently retains the polymerase on the template during transient thermal fluctuations when it might otherwise dissociate.

The  $\beta_2$  bracelet cannot spontaneously associate with high molecular weight DNA, it requires a multiprotein DnaX-complex to open and close it around DNA using the energy of ATP hydrolysis (Wickner, Proc. Natl. Acad. Sci. USA 73:35411-3515 [1976]; Naktinis *et al.*, J. Biol. Chem., 270:13358-13365 [1985]; and Dallmann *et al.*, J. Biol. Chem., 270:29555-29562 [1995]). In *E. coli*, the *dnaX* gene encodes two proteins,  $\tau$  and  $\gamma$ .  $\gamma$  is generated by a programmed ribosomal frameshifting mechanism five-sevenths of the way through *dnaX* mRNA, placing the ribosome in a -1 reading frame where it immediately encounters a stop codon (Flower and McHenry Proc. Natl. Acad. Sci. USA 87:3713-3717 [1990]; Blinkowa and Walker, Nucl. Acids Res.,

- 3 -

18:1725-1729 [1990]; and Tsuchihashi and Kornberg, Proc. Natl. Acad. Sci. USA 87:2516-2520 [1990]). In *E. coli*, the DnaX-complex has the stoichiometry  $\gamma_2\tau_2\delta_1\delta'_1\chi_1\psi_1$  (Dallmann and McHenry, J. Biol. Chem., 270:29563-29569 [1995]). The  $\tau$  protein contains an additional carboxyl-terminal domain that interacts tightly with the polymerase, holding two polymerases together in one complex that can coordinately replicate the leading and lagging strand of the replication fork simultaneously (McHenry, J. Biol. Chem., 257:2657-2663 [1982]; Studwell and O'Donnell, Biol. Chem., 266:19833-19841 [1991]; McHenry, Ann. Rev. Biochem. 57:519-550 [1988]).

Conservation of a frameshifting mechanism to generate related ATPases is significant in that, by analogy to *E. coli*, can both assemble a processivity factor onto primed DNA. In *E. coli*, ribosomes frameshift at the sequence A AAA AAG into a -1 frame where the lysine UUU anticodon tRNA can base pair with 6As before elongating (Flower and McHenry, Proc. Natl. Acad. Sci. USA 87:3713-3717 [1990]; Blinkowa and Walker, Nucleic Acids Res., 18:1725-1729 [1990]; and Tsuchihashi and Kornberg, Proc. Natl. Acad. Sci. USA 87:2516-2520 [1990]).

Pol IIIs are apparently conserved throughout mesophilic eubacteria. In addition to *E. coli* and related proteobacteria, the enzyme has been purified from the firmicute *Bacillus subtilis* (Low *et al.*, J. Biol. Chem., 251:1311-1325 [1976]; Hammond and Brown [1992]). With the proliferation of bacterial genomes sequenced, by inference from DNA sequence, pol III exists in organisms as widely divergent as *Caulobacter*, *Mycobacteria*, *Mycoplasma*, *B. subtilis* and *Synechocystis*. The existence of *dnaX* and *dnaN* (structural gene for  $\beta$ ) is also apparent in these organisms. These general replication mechanisms are conserved even more broadly in biology. Although eukaryotes do not contain polymerases homologous to pol III, eukaryotes contain special polymerases devoted to chromosomal replication and  $\beta$ -like processivity factors (PCNA) and DnaX-like ATPases (RFC, Activator I) that assemble these processivity factors on DNA (Yoder and Burgers, J. Biol.



- 4 -

Chem., 266:22689-22697 [1991]; Brush and Stillman, Meth. Enzymol., 262:522-548 [1995]; Uhlmann *et al.*, Proc. Natl. Acad. Sci. USA 93:6521-6526 [1996]).

Helicases serve a variety of functions in DNA metabolism. Cellular (*E. coli* dnaB, priA, and rep proteins), phage (T4 gene 41 and dda proteins; T7 gene 4 protein), and viral (SV40 T antigen; HSV-1 UL5/UL52 complex and UL9 protein) helicases are involved in the initiation of replication, by unwinding DNA so that other proteins of the replication complex can assemble on the ssDNA. These proteins also participate in the elongation phase of replication, by unwinding the duplex DNA ahead of this complex to provide the required template. Other helicases (e.g., the *E. coli* recBCD and recQ proteins) are implicated in recombination by genetic criteria. Another class of helicases includes the *E. coli* uvrAB and uvrD. These helicases act in nucleotide excision repair or methyl-directed mismatch repair during both pre-incision (recognition of DNA damage or alteration) and post-incision (displacement of damaged fragment) steps. See, for example, USPN 5,747,247.

DNA mispairing can occur *in vivo* and is recognized and corrected by repair proteins. Mismatch repair has been studied most intensively in *E. coli*, *Salmonella typhimurium*, and *S. pneumoniae*. The MutS, MutH and MutL proteins of *E. coli* are involved in the repair of DNA mismatches, as is the product of the *uvrD* gene in *E. coli*, helicase II. See, for example, USPN 5,750,335.

The best defined mismatch repair pathway is the *E. coli* MutHLS pathway that promotes a long-patch (approximately 3 Kb) excision repair reaction which is dependent on the mutH, mutL, mutS and mutU (*uvrD*) gene products. The MutHLS pathway appears to be the most active mismatch repair pathway in *E. coli* and is known to both increase the fidelity of DNA replication and to act on recombination intermediates containing mispaired bases. The system has been reconstituted *in vitro*, and requires the mutH, mutL, mutS and *uvrD* (helicase II) proteins along with DNA polymerase III

- 5 -

holoenzyme, DNA ligase, single-stranded DNA binding protein (SSB) and one of the single-stranded DNA exonucleases, Exo I, Exo VII or RecJ. A similar pathway in yeast includes the yeast MSH2 gene and two mutL-like genes referred to as PMS1 and MLH1. See, for example, USPN 6,191,268.

5           The *E. coli* bacterial Uvr proteins are capable of excising damaged DNA sites caused by a broad spectrum of chemical agents that distort the backbone geometry of the DNA double helix. As a result, if the DNA were damaged by chemicals in the environmental sample, the Uvr proteins will cleave and excise the damaged region. Subsequent resynthesis by DNA  
10       polymerase I will incorporate labeled or unlabeled nucleotides into the DNA. See, for example, USPN 6,060,288.

          Replication of the lagging strand of DNA is mediated by a multiprotein complex composed of proteins priA, dnaT, dnaB, dnaC, and dnaG. This complex is referred to as a primosome. Purified priA has ATPase, helicase,  
15       translocase, and primosome assembly activities. This gene may be essential in recombination and DNA repair since it binds to D-loops, interacts with recG and has helicase activity. The 3'-5' DNA helicase activity of priA inhibits recombination. See, for example, USPN 6,146,846.

## 20                           BRIEF SUMMARY OF THE INVENTION

          The invention is directed to an isolated polypeptide wherein said polypeptide comprises an amino acid sequence having at least 95% sequence identity to the amino acid sequence of SEQ ID NO: (uvrD helicase) 68.

25           The invention is directed to an isolated polypeptide wherein said polypeptide comprises an amino acid sequence having at least 95% sequence identity to the amino acid sequence of SEQ ID NO: (DNA-G Primase) 72.

          The invention is directed to an isolated polypeptide wherein said polypeptide comprises an amino acid sequence having at least 95% sequence  
30       identity to the amino acid sequence of SEQ ID NO: (priA helicase) 76.

- 6 -

The invention is directed to an isolated polypeptide wherein said polypeptide comprises an amino acid sequence having at least 95% sequence identity to the amino acid sequence of SEQ ID NO: (delta subunit) 10.

5 The invention is directed to an isolated polypeptide wherein said polypeptide comprises an amino acid sequence having at least 95% sequence identity to the amino acid sequence of SEQ ID NO: (delta prime subunit) 17.

The invention is directed to an isolated polypeptide wherein said polypeptide comprises an amino acid sequence having at least 95% sequence identity to the amino acid sequence of SEQ ID NO: (beta subunit) 23.

10 The invention is directed to an isolated polypeptide wherein said polypeptide comprises an amino acid sequence having at least 95% sequence identity to the amino acid sequence of SEQ ID NO: (ssb protein) 32.

15 The invention is directed to an isolated polypeptide wherein said polypeptide comprises an amino acid sequence having at least 95% sequence identity to the amino acid sequence of SEQ ID NO: (epsilon-1, dnaQ-1) 37.

The invention is directed to an isolated polypeptide wherein said polypeptide comprises an amino acid sequence having at least 95% sequence identity to the amino acid sequence of SEQ ID NO: (epsilon-2, dnaQ-2) 82.

20 The invention is directed to a method of producing a polypeptide encoded by a nucleotide sequence, wherein said polypeptide comprises an amino acid sequence having at least 95% sequence identity to the amino acid sequence of one of SEQ ID NOS: 68, 72, 76, 10, 17, 23, 32, 37, and 82, comprising culturing a host cell comprising said nucleotide sequence under conditions such that said polypeptide is expressed, and recovering said  
25 polypeptide.

The invention is directed to a method of synthesizing DNA which comprises utilizing one or more polypeptides, said one or more polypeptides comprising an amino acid sequence having at least 95% sequence identity to an amino acid sequence selected from the group consisting of SEQ ID NOS:  
30 68, 72, 76, 10, 17, 23, 32, 37 and 82.

- 7 -

Further objects and advantages of the present invention will be clear from the description that follows.

#### BRIEF DESCRIPTION OF THE FIGURES

5

In all of the following Figures that show alignments (DNA or amino acids), the "+" indicates similar, but not identical residues. In the DNA sequences with underlined regions, unless otherwise indicated, the underlining indicates bases generated by the degenerate primers used to generate the DNA of interest. Also unless otherwise indicated, the sequences between the sequences generated by the primers were used in the searches to generate deduced amino acid sequences (*i.e.*, the primer-generated sequences were excluded from the searches).

10

FIG. 1. Protein concentration profile of  $\text{Ni}^{++}$ -NTA column purification of N-terminal tagged *T. thermophilus*  $\alpha$ .

15

FIG. 2. SDS-PAGE analysis of expression optimization of pTAC-CCA-TE.

FIG. 3. Protein concentration profile of ammonium sulfate precipitation optimization of native *T. thermophilus*  $\alpha$ .

20

FIG. 4 SDS-PAGE analysis of ammonium sulfate precipitation optimization of *T. thermophilus*  $\alpha$ .

FIG. 5. Activity assay analysis of ammonium sulfate precipitation optimization of *T. thermophilus*  $\alpha$  using the gap-filling assay.

FIG. 6. SDS-polyacrylamide summary gel of the different purification steps of native *T. thermophilus* expressed as a translationally coupled protein.

25

FIG. 7. Biotin blot analysis of the growth optimization for expression of N-terminal tagged *T. thermophilus* DnaX subunits from pA1-NB-TX/AP1.L1.

FIG. 8. Protein concentration profile of the fractions from the  $\text{Ni}^{++}$ -NTA column purification of N-terminal tagged *T. thermophilus* DnaX.

30

- 8 -

FIGs. 9A and B. SDS-PAGE analysis of the fraction from the  $\text{Ni}^{++}$ -NTA column purification of N-terminal tagged *T. thermophilus* DnaX.

FIGs. 10A and B. SDS-PAGE analysis of the fraction from the avidin column purification of N-terminal tagged *T. thermophilus* DnaX.

5        FIG. 11. Western analysis of various antiserum dilutions for determination of dilutions to use in *T. thermophilus* DnaX detection.

FIG. 12. Western analysis of various *T. thermophilus* DnaX dilutions for determination of the limit of DnaX detection at antiserum dilution of 1:6400.

10        FIG. 13 The DNA sequence (SEQ ID NO:9) of the *T. thermophilus* *holA* gene ( $\delta$  subunit).

FIG. 14. The amino acid sequence (SEQ ID NO: 10) of *T. thermophilus*  $\delta$ -subunit (*holA* gene).

15        FIG. 15. Alignment of the amino acid sequence of  $\delta$  from *T. thermophilus* and *E. coli*.

FIG. 16 Alignment of the amino acid sequence of  $\delta$ -subunit from *A. aerolicus*, *T. thermophilus*, *B. subtilis*, *E. coli* and *H. influenzae*.

FIG. 17 Biotin blot analysis of growth/induction time optimization of expression of *T. thermophilus*  $\delta$  by pA1-NB-TD/AP1.L1.

20        FIG. 18. Optimization of precipitation of *T. thermophilus*  $\delta$  by ammonium sulfate.

FIGs. 19A and B. SDS-PAGE analysis of fractions from the  $\text{Ni}^{++}$ -NTA column purification of *T. thermophilus*  $\delta$ .

25        FIG. 20. Protein concentration profile of fractions from the avidin column purification of *T. thermophilus*  $\delta$ .

FIG. 21 SDS-PAGE analysis of fractions from the avidin column purification of *T. thermophilus*  $\delta$ .

FIG. 22. The DNA sequence (SEQ ID NO: 16 ) of the *T. thermophilus* *holB* gene encoding the  $\delta'$ -subunit of the *T. thermophilus* Pol III holoenzyme.

- 9 -

FIG. 23. The amino acid sequence (SEQ ID NO:17) of the *T. thermophilus*  $\delta'$ -subunit derived from the DNA sequence of the *T. thermophilus* *holB* gene.

FIG. 24. Alignment of the amino acid sequence comparing *E. coli* and  
5 *T. thermophilus*  $\delta'$ .

FIG. 25. Alignment of the amino acid sequence of  $\delta'$ -subunit from *A. aerolicus*, *T. thermophilus*, *B. subtilis*, *E. coli* and *H. influenzae* and *Rickettsia*.

FIG. 26. Biotin blot analysis of growth/induction time optimization of  
10 expression of *T. thermophilus*  $\delta'$  by pA1-NB-TD'/AP1.L1.

FIGS. 27A and B. SDS-PAGE Analysis  $\text{Ni}^{++}$ -NTA column purification of N-terminal tagged *T. thermophilus*  $\delta'$ .

FIG. 28. Protein concentration profile of fractions eluting from the Sephacryl S-300 gel filtration column purification of *T. thermophilus*  $\delta'$ .

15 FIG. 29. SDS-PAGE analysis of fractions from the Sephacryl S-300 column purification of *T. thermophilus*  $\delta'$ .

FIG. 30. SDS-PAGE summary of the purification of *T. thermophilus*  $\delta'$  as a translationally coupled protein.

FIG. 31. Biotin blot analysis of growth/induction time optimization at  
20 different temperatures of expression of *T. thermophilus*  $\beta$  by pA1-NB-TN/AP1.L1.

FIG. 32. Protein concentration profile of fractions eluting from the  $\text{Ni}^{++}$ -NTA column purification of *T. thermophilus*  $\beta$ .

FIG. 33. Primer extension assay to determine stimulation of *T. thermophilus*  $\alpha$  by *T. thermophilus*  $\beta$ .  
25

FIG. 34. Protein concentration profile of fractions eluting from a Sephacryl S-300 gel filtration column purification of *T. thermophilus*  $\beta$ .

FIGS. 35A and B. SDS-PAGE analysis of fractions eluting from a Sephacryl S-300 gel filtration column purification of *T. thermophilus*  $\beta$ .

- 10 -

FIG. 36. The pooled fractions of *T. thermophilus*  $\beta$  from the Sephacryl S-300 gel filtration column that was used in production of antibodies directed against  $\beta$ .

FIG. 37. Western analysis of various antiserum dilutions for determination of dilutions to use in *T. thermophilus*  $\beta$  detection.

FIG. 38. Western analysis of various *T. thermophilus*  $\beta$  dilutions for determination of the limit of  $\beta$  detection at antiserum dilution of 1:6400.

FIG. 39. M13gori reconstitution of *T. thermophilus* Pol III subunits.

FIG. 40. Temperature dependence for a functional *T. thermophilus* holoenzyme in the reconstitution assay.

FIG. 41. The reconstitution assay in which *T. thermophilus* A.  $\alpha$  , B.  $\tau/\gamma$  , C.  $\beta$  , D.  $\delta$  , and E.  $\delta'$  is/are titrated while the other subunits are held constant.

FIG. 42. Reconstitution assay in the absence of all subunits except  $\alpha$  to determine the background activity present due to spurious binding of  $\alpha$  alone to the template and extending the primer a short distance at each binding event.

FIG. 43. Reconstitution assay in the absence of  $\beta$ , but in the presence of the other subunits, to determine the effect of the other subunits on background activity present due to spurious binding of  $\alpha$ .

FIGs. 44A-E. Sephacryl S-200 gel filtration of subunits of the clamp loading complex showing protein-protein interactions.

FIGs. 45A-C. Sephacryl S-200 gel filtration of *T. thermophilus*  $\alpha$  with the subunits of the clamp loading complex showing protein-protein interactions.

FIG. 46. Sephacryl S-200 gel filtration of *T. thermophilus*  $\beta$ .

FIG. 47. The DNA sequence (SEQ ID NO: 31) of the gene encoding *T. thermophilus* SSB.

FIG. 48. The amino acid sequence of (SEQ ID NO:32) the *T. thermophilus* SSB protein.

- 11 -

FIG. 49. Sequence alignment of *T. thermophilus* SSB compared with SSB amino acid sequences from *Aquifex*, *B. subtilis*, *E. coli* and *H. influenzae*.

FIG. 50. Sequence alignment of the N-terminal region of *T. thermophilus* SSB with the C-terminal region of *T. thermophilus* SSB.

5 FIG. 51. Biotin blot analysis of relevant fractions from the Ni<sup>++</sup>-NTA column purification of *T. thermophilus* SSB.

FIG. 52. The DNA sequence of the gene encoding *T. thermophilus* epsilon-1 ( $\epsilon$ -1, *dnaQ*-1)(SEQ ID NO:36).

10 FIG. 53. The amino acid sequence (SEQ ID NO:37) of a *T. thermophilus* epsilon-1 subunit ( $\epsilon$ -1).

FIG. 54. The DNA sequence (SEQ ID NO:67) of the gene encoding *T. thermophilus uvrD*.

FIG. 55. The amino acid sequence (SEQ ID NO:68) of a *T. thermophilus uvrD* protein.

15 FIG. 56. The DNA sequence (SEQ ID NO:71) of a *T. thermophilus dnaG* gene.

FIG. 57. The amino acid sequence (SEQ ID NO:72) of a *T. thermophilus dnaG* protein.

20 FIG. 58. The DNA sequence (SEQ ID NO:75) of a *T. thermophilus priA* gene.

FIG. 59. The amino acid sequence (SEQ ID NO:76) of a *T. thermophilus priA* protein.

FIG. 60. The DNA sequence (SEQ ID NO: 81) of a *T. thermophilus dnaQ*-2 gene ( $\epsilon$ 2 subunit).

25 FIG. 61. The amino acid sequence (SEQ ID NO: 82) of a *T. thermophilus*  $\epsilon$ 2 subunit.

FIG. 62. The DNA sequence (SEQ ID NO: 22) of a *T. thermophilus dnaN* gene ( $\beta$  subunit).

30 FIG. 63. The amino acid sequence (SEQ ID NO: 23) of a *T. thermophilus*  $\beta$  subunit.



## DETAILED DESCRIPTION OF THE INVENTION

## A. Definitions

5 In order to provide a clear and consistent understanding of the specification and claims, including the scope to be given such terms, the following definitions are provided. It is also to be noted that the term "a" or "an" entity, refers to one or more of that entity, for example, "a polynucleotide," is understood to represent one or more polynucleotides.

10 As used herein, the term "DNA polymerase III holoenzyme" refers to the entire DNA polymerase III entity (*i.e.*, all of the polymerase subunits, as well as the other associated accessory proteins, such as ssb, dnaG, uvrD and priA, required for processive replication of a chromosome or genome), while "DNA polymerase III" is just the core ( $\alpha$ ,  $\epsilon$ ,  $\theta$ ). "DNA polymerase III holoenzyme subunit" is used in reference to any of the subunit entities that  
15 comprise the DNA polymerase III holoenzyme. Thus, the term "DNA polymerase III" encompasses "DNA polymerase III holoenzyme subunits" and "DNA polymerase III subunits."

20 The term "5' exonuclease activity" refers to the presence of an activity in a protein which is capable of removing nucleotides from the 5' end of an oligonucleotide. 5' exonuclease activity may be measured using any of the assays provided herein.

25 The term "3' exonuclease activity" refers to the presence of an activity in a protein which is capable of removing nucleotides from the 3' end of an oligonucleotide. 3' exonuclease activity may be measured using any of the assays provided herein.

30 The terms "DNA polymerase activity," "synthetic activity" and "polymerase activity" are used interchangeably and refer to the ability of an enzyme to synthesize new DNA strands by the incorporation of deoxynucleoside triphosphates. The examples below provide assays for the measurement of DNA polymerase activity. A protein which can direct the

- 13 -

synthesis of new DNA strands (DNA synthesis) by the incorporation of deoxynucleoside triphosphates in a template-dependent manner is said to be "capable of DNA synthetic activity."

5 A DNA synthesis terminating agent which terminates DNA synthesis at a specific nucleotide base refers to compounds, including but not limited to, dideoxynucleosides having a 2', 3' dideoxy structure (*e.g.*, ddATP, ddCTP, ddGTP and ddTTP). Any compound capable of specifically terminating a DNA sequencing reaction at a specific base may be employed as a DNA synthesis terminating agent.

10 The term "gene" refers to a nucleic acid (*e.g.*, DNA) sequence that comprises coding sequences necessary for the production of a polypeptide or precursor (*e.g.*, DNA polymerase III holoenzyme, holoenzyme subunit, or accessory protein as appropriate). The polypeptide can be encoded by a full length coding sequence or by any portion of the coding sequence so long as  
15 the desired activity or functional properties (*e.g.*, enzymatic activity, ligand binding, signal transduction, etc.) of the full-length polypeptide or fragment are retained. The term also encompasses the coding region of a structural gene and includes sequences located adjacent to the coding region on both the 5' and 3' ends for a distance of about 1 kb on either end such that the gene  
20 corresponds to the length of the full-length mRNA.

The term "gene" encompasses both cDNA and genomic forms of a gene. A genomic form or clone of a gene contains the coding region interrupted with non-coding sequences termed "intervening regions" or "intervening sequences." The mRNA functions during translation to specify  
25 the sequence or order of amino acids in a nascent polypeptide.

In particular, the terms "DNA polymerase III holoenzyme" and "holoenzyme subunit gene" refer to the full-length DNA polymerase III holoenzyme, and holoenzyme subunit nucleotide sequence(s), respectively. However, it is also intended that the term encompass fragments of the DNA  
30 polymerase III holoenzyme and holoenzyme subunit sequences, such as those that encode particular domains of interest, including subunit proteins, as well

- 14 -

as other domains within the full-length DNA polymerase III holoenzyme or holoenzyme subunit nucleotide sequence. Furthermore, the terms "DNA polymerase III holoenzyme," "holoenzyme subunit nucleotide sequence," "DNA polymerase III holoenzyme," and "holoenzyme subunit polynucleotide sequence" encompasses DNA, cDNA, and RNA (*e.g.*, mRNA) sequences.

As used herein, the term "accessory protein(s)" refers to a protein or polypeptide required for, or involved in, processive replication of a chromosome or genome. The term further encompasses the full length polypeptide or protein. Where fragments of accessory proteins are intended, the fragment of the polypeptide or protein will be clearly indicated.

"Where "amino acid sequence" is recited herein to refer to an amino acid sequence of a naturally occurring protein molecule, "amino acid sequence" and like terms, such as "polypeptide" or "protein" are not meant to limit the amino acid sequence to the complete, native amino acid sequence associated with the recited proteins. Further, "polypeptide" and "protein" are used interchangeably unless clearly indicated otherwise. Where a distinction between "polypeptide" and "protein" is intended, such will be made clear.

Genomic forms of a gene may also include sequences located on both the 5' and 3' end of the sequences which are present on the RNA transcript. These sequences are referred to as "flanking" sequences or regions (these flanking sequences are located 5' or 3' to the translated sequences present on the mRNA transcript). The 5' flanking region may contain regulatory sequences such as promoters and enhancers which control or influence the transcription of the gene. The 3' flanking region may contain sequences which direct the termination of transcription, post-transcriptional cleavage and polyadenylation.

The term "wild-type" refers to a gene or gene product which has the characteristics of that gene or gene product when isolated from a naturally occurring source. A wild-type gene is that which is most frequently observed in a population and is thus arbitrarily designated the "normal" or "wild-type" form of the gene. In contrast, the term "modified" or "mutant" refers to a gene

- 15 -

or gene product which displays modifications in sequence and or functional properties (*i.e.*, altered characteristics) when compared to the wild-type gene or gene product. It is noted that naturally-occurring mutants can be isolated; these are identified by the fact that they have altered characteristics when compared to the wild-type gene or gene product.

The terms "nucleotide sequence encoding," "nucleic acid molecule encoding," "DNA sequence encoding," and "DNA encoding" refer to the order or sequence of deoxyribonucleotides along a strand of deoxyribonucleic acid. The order of these deoxyribonucleotides determines the order of amino acids along the polypeptide (protein) chain. The DNA sequence thus codes for the amino acid sequence.

The term "oligonucleotide" is defined as a molecule comprised of two or more deoxyribonucleotides or ribonucleotides, preferably more than three, and usually more than ten. The exact size will depend on many factors, which in turn depends on the ultimate function or use of the oligonucleotide. The oligonucleotide may be generated in any manner, including chemical synthesis, DNA replication, reverse transcription, PCR, or a combination thereof.

Because mononucleotides are reacted to make oligonucleotides in a manner such that the 5' phosphate of one mononucleotide pentose ring is attached to the 3' oxygen of its neighbor in one direction via a phosphodiester linkage, an end of an oligonucleotide is referred to as the "5' end" if its 5' phosphate is not linked to the 3' oxygen of a mononucleotide pentose ring and as the "3' end" if its 3' oxygen is not linked to a 5' phosphate of a subsequent mononucleotide pentose ring. As used herein, a nucleic acid sequence, even if internal to a larger oligonucleotide, also may be said to have 5' and 3' ends.

When two different, non-overlapping oligonucleotides anneal to different regions of the same linear complementary nucleic acid sequence, and the 3' end of one oligonucleotide points towards the 5' end of the other, the former may be called the "upstream" oligonucleotide and the latter the "downstream" oligonucleotide. In either a linear or circular DNA molecule,

- 16 -

discrete elements are referred to as being "upstream" or 5' of the "downstream" or 3' elements. This terminology reflects the fact that transcription proceeds in a 5' to 3' fashion along the DNA strand. The promoter and enhancer elements which direct transcription of a linked gene are generally located 5' or upstream of the coding region. However, enhancer elements can exert their effect even when located 3' of the promoter element and the coding region. Transcription termination and polyadenylation signals are located 3' or downstream of the coding region.

The term "coding region" when used in reference to a structural gene refers to the nucleotide sequences which encode the amino acids found in the nascent polypeptide as a result of translation of a mRNA molecule. The coding region is bounded on the 5' side by the nucleotide triplet "ATG" which encodes the initiator methionine and on the 3' side by one of the three triplets which specify stop codons (*i.e.*, TAA, TAG, TGA). Occasionally, the ATG is replaced by GTG.

The term "polynucleotide molecule comprising a nucleotide sequence encoding a gene," means a nucleic acid sequence comprising the coding region of a gene or in other words the nucleic acid sequence which encodes a gene product. The coding region may be present in either a cDNA, genomic DNA or RNA form. When present in a DNA form, the polynucleotide may be single-stranded (*i.e.*, the sense strand) or double-stranded. Suitable control elements such as enhancers/promoters, splice junctions, polyadenylation signals, *etc.* may be placed in close proximity to the coding region of the gene if needed to permit proper initiation of transcription and/or correct processing of the primary RNA transcript. Alternatively, the coding region utilized in the expression vectors of the present invention may contain endogenous enhancers/promoters, splice junctions, intervening sequences, polyadenylation signals, *etc.*, or a combination of both endogenous and exogenous control elements.

The term "regulatory element" refers to a genetic element which controls some aspect of the expression of nucleic acid sequences. For

- 17 -

example, a promoter is a regulatory element which facilitates the initiation of transcription of an operably linked coding region. Other regulatory elements are splicing signals, polyadenylation signals, termination signals, etc. (defined *infra*).

5 Transcriptional control signals in eukaryotes comprise "promoter" and "enhancer" elements. Promoters and enhancers consist of short arrays of DNA sequences that interact specifically with cellular proteins involved in transcription (Maniatis *et al.*, Science 236:1237 [1987]). Promoter and enhancer elements have been isolated from a variety of eukaryotic sources including genes in yeast, insect and mammalian cells and viruses (analogous control elements, *i.e.*, promoters, are also found in prokaryotes). The selection of a particular promoter and enhancer depends on what cell type is to be used to express the protein of interest. Some eukaryotic promoters and enhancers have a broad host range while others are functional in a limited subset of cell types (for review see, Voss *et al.*, Trends Biochem. Sci., 11:287 [1986]; and Maniatis *et al.*, *supra*). For example, the SV40 early gene enhancer is very active in a wide variety of cell types from many mammalian species and has been widely used for the expression of proteins in mammalian cells (Dijkema *et al.*, EMBO J. 4:761 [1985]). Two other examples of promoter/enhancer elements active in a broad range of mammalian cell types are those from the human elongation factor 1 $\alpha$  gene (Uetsuki *et al.*, J. Biol. Chem., 264:5791 [1989]; Kim *et al.*, Gene 91:217 [1990]; and Mizushima and Nagata, Nucl. Acids. Res., 18:5322 [1990]) and the long terminal repeats of the Rous sarcoma virus (Gorman *et al.*, Proc. Natl. Acad. Sci. USA 79:6777 [1982]) and the human cytomegalovirus (Boshart *et al.*, Cell 41:521 [1985]).

The term "promoter/enhancer" denotes a segment of DNA which contains sequences capable of providing both promoter and enhancer functions (*i.e.*, the functions provided by a promoter element and an enhancer element, see above for a discussion of these functions). For example, the long terminal repeats of retroviruses contain both promoter and enhancer functions. The enhancer/promoter may be "endogenous" or "exogenous" or

- 18 -

"heterologous." An "endogenous" enhancer/promoter is one which is naturally linked with a given gene in the genome. An "exogenous" or "heterologous" enhancer/promoter is one which is placed in juxtaposition to a gene by means of genetic manipulation (*i.e.*, molecular biological techniques) such that transcription of that gene is directed by the linked enhancer/promoter. Many promoter/enhancer sequences can be used to express the proteins of the invention.

Efficient expression of recombinant DNA sequences in eukaryotic cells requires expression of signals directing the efficient termination and polyadenylation of the resulting transcript. Transcription termination signals are generally found downstream of the polyadenylation signal and are a few hundred nucleotides in length. The term "poly A site" or "poly A sequence" denotes a DNA sequence which directs both the termination and polyadenylation of the nascent RNA transcript. Efficient polyadenylation of the recombinant transcript is desirable as transcripts lacking a poly A tail are unstable and are rapidly degraded. The poly A signal utilized in an expression vector may be "heterologous" or "endogenous." An endogenous poly A signal is one that is found naturally at the 3' end of the coding region of a given gene in the genome. A heterologous poly A signal is one which is isolated from one gene and placed 3' of another gene. A commonly used heterologous poly A signal is the SV40 poly A signal. The SV40 poly A signal is contained on a 237 bp *Bam*HI/*Bcl*II restriction fragment and directs both termination and polyadenylation (Sambrook, *supra*, at 16.6-16.7).

As used herein, the term "vector" is used in reference to nucleic acid molecules that transfer DNA segment(s) from one cell to another. The term "vehicle" is sometimes used interchangeably with "vector." "Vector" is also used interchangeably with "plasmid." Where a difference is intended, the difference will be made clear.

The term "expression vector" refers to a recombinant DNA molecule containing a desired coding sequence and appropriate nucleic acid sequences necessary for the expression of the operably linked coding sequence" in a

- 19 -

particular host organism. Nucleic acid sequences necessary for expression in prokaryotes usually include a promoter, an operator (optional), and a ribosome binding site, often along with other sequences. Eukaryotic cells are known to utilize promoters, enhancers, and termination and polyadenylation signals.

5           The term "transformation" as used herein refers to the introduction of foreign DNA into eukaryotic cells. Transformation may be accomplished by a variety of means known to the art including calcium phosphate-DNA co-precipitation, DEAE-dextran-mediated transfection, polybrene-mediated transfection, transfection, electroporation, microinjection, liposome fusion, lipofection, protoplast fusion, retroviral infection, and biolistics.

10           The term "selectable marker" refers to the use of a gene which encodes an enzymatic activity that confers the ability to grow in medium lacking what would otherwise be an essential nutrient (e.g. the *HIS3* gene in yeast cells); in addition, a selectable marker may confer resistance to an antibiotic or drug upon the cell in which the selectable marker is expressed. Selectable markers may be "dominant"; a dominant selectable marker encodes an enzymatic activity that can be detected in any eukaryotic cell line. Examples of dominant selectable markers include the bacterial aminoglycoside 3' phosphotransferase gene (also referred to as the *neo* gene) which confers resistance to the drug G418 in mammalian cells, the bacterial hygromycin G phosphotransferase (20 *hyg*) gene which confers resistance to the antibiotic hygromycin and the bacterial xanthine-guanine phosphoribosyl transferase gene (also referred to as the *gpt* gene) which confers the ability to grow in the presence of mycophenolic acid. Other selectable markers are not dominant in that their use must be in conjunction with a cell line that lacks the relevant enzyme activity. Examples of non-dominant selectable markers include the thymidine kinase (*tk*) gene which is used in conjunction with *tk*<sup>-</sup> cell lines, the CAD gene which is used in conjunction with CAD-deficient cells and the mammalian hypoxanthine-guanine phosphoribosyl transferase (*hprt*) gene which is used in conjunction with *hprt*<sup>-</sup> cell lines. A review of the use of selectable markers in mammalian cell lines is provided in Sambrook *et al.*, *Molecular Cloning: A*



- 20 -

*Laboratory Manual*, 2nd ed., Cold Spring Harbor Laboratory Press, New York (1989) pp.16.9-16.15.

Eukaryotic expression vectors may also contain "viral replicons" or "viral origins of replication." Viral replicons are viral DNA sequences which allow for the extrachromosomal replication of a vector in a host cell expressing the appropriate replication factors. Vectors which contain either the SV40 or polyoma virus origin of replication replicate to high copy number. Vectors which contain the replicons from bovine papillomavirus or Epstein-Barr virus replicate extrachromosomally at low copy number (~100 copies/cell).

The thermophilic DNA polymerase III holoenzyme or holoenzyme subunits or accessory proteins (for example, dnaG, priA, uvrD) may be expressed in either prokaryotic or eukaryotic host cells. Nucleic acid encoding the thermophilic DNA polymerase III holoenzyme or holoenzyme subunit or accessory proteins (for example, dnaG, priA, uvrD) may be introduced into bacterial host cells by a number of means including transformation of bacterial cells made competent for transformation by treatment with calcium chloride or by electroporation. If the thermophilic DNA polymerase III holoenzyme or holoenzyme subunit or accessory proteins (for example, dnaG, priA, uvrD) are to be expressed in eukaryotic host cells, nucleic acid encoding the thermophilic DNA polymerase III holoenzyme or holoenzyme subunit or accessory proteins (for example, dnaG, priA, uvrD) may be introduced into eukaryotic host cells by a number of means including calcium phosphate co-precipitation, spheroplast fusion, electroporation and the like. When the eukaryotic host cell is a yeast cell, transformation may be affected by treatment of the host cells with lithium acetate or by electroporation or any other method known in the art. It is contemplated that any host cell will be useful in producing the peptides or proteins or fragments thereof of the invention.

"Hybridization" methods involve the annealing of a complementary sequence to the target nucleic acid (the sequence to be detected). The ability

- 21 -

of two polymers of nucleic acid containing complementary sequences to find each other and anneal through base pairing interaction is a well-recognized phenomenon. The initial observations of the "hybridization" process by Marmur and Lane, (*See e.g.*, Marmur and Lane, Proc. Natl. Acad. Sci. USA 46:453 [1960]); and Doty *et al.*, Proc. Natl. Acad. Sci. USA 46:461 [1960]) have been followed by the refinement of this process into an essential tool of modern biology. Nonetheless, a number of problems have prevented the wide scale use of hybridization as a tool in diagnostics. Among the more formidable problems are: 1) the inefficiency of hybridization; 2) the low concentration of specific target sequences in a mixture of genomic DNA; and 3) the hybridization of only partially complementary probes and targets.

With regard to efficiency, it is experimentally observed that only a fraction of the possible number of probe-target complexes are formed in a hybridization reaction. This is particularly true with short oligonucleotide probes (less than 100 bases in length). There are three fundamental causes: a) hybridization cannot occur because of secondary and tertiary structure interactions; b) strands of DNA containing the target sequence have rehybridized (reannealed) to their complementary strand; and c) some target molecules are prevented from hybridization when they are used in hybridization formats that immobilize the target nucleic acids to a solid surface.

Even where the sequence of a probe is completely complementary to the sequence of the target (*i.e.*, the target's primary structure), the target sequence must be made accessible to the probe via rearrangements of higher-order structure. These higher-order structural rearrangements may concern either the secondary structure or tertiary structure of the molecule. Secondary structure is determined by intramolecular bonding. In the case of DNA or RNA targets this consists of hybridization within a single, continuous strand of bases (as opposed to hybridization between two different strands). Depending on the extent and position of intramolecular bonding, the probe can be displaced from the target sequence preventing hybridization.

- 22 -

Solution hybridization of oligonucleotide probes to denatured double-stranded DNA is further complicated by the fact that the longer complementary target strands can renature or reanneal. Again, hybridized probe is displaced by this process. This results in a low yield of hybridization (low "coverage") relative to the starting concentrations of probe and target.

With regard to low target sequence concentration, the DNA fragment containing the target sequence is usually in relatively low abundance in genomic DNA. This presents great technical difficulties; most conventional methods that use oligonucleotide probes lack the sensitivity necessary to detect hybridization at such low levels.

One attempt at a solution to the target sequence concentration problem is the amplification of the detection signal. Most often this entails placing one or more labels on an oligonucleotide probe. In the case of non-radioactive labels, even the highest affinity reagents have been found to be unsuitable for the detection of single copy genes in genomic DNA with oligonucleotide probes. (*See, Wallace et al., Biochimie 67:755 [1985]*). In the case of radioactive oligonucleotide probes, only extremely high specific activities are found to show satisfactory results. (*See, Studencki and Wallace, DNA 3:1 [1984]; and Studencki et al., Human Genetics 37:42 [1985]*).

With regard to complementarity, it is important for some diagnostic applications to determine whether the hybridization represents complete or partial complementarity. For example, where it is desired to detect simply the presence or absence of pathogen DNA (such as from a virus, bacterium, fungi, mycoplasma, protozoan) it is only important that the hybridization method ensures hybridization when the relevant sequence is present; conditions can be selected where both partially complementary probes and completely complementary probes will hybridize. Other diagnostic applications, however, may require that the hybridization method distinguish between partial and complete complementarity. It may be of interest to detect genetic polymorphisms. For example, human hemoglobin is composed, in part, of four polypeptide chains. Two of these chains are identical chains of 141

- 23 -

amino acids (alpha chains) and two of these chains are identical chains of 146 amino acids (beta chains). The gene encoding the beta chain is known to exhibit polymorphism. The normal allele encodes a beta chain having glutamic acid at the sixth position. The mutant allele encodes a beta chain having valine at the sixth position. This difference in amino acids has a profound (most profound when the individual is homozygous for the mutant allele) physiological impact known clinically as sickle cell anemia. It is well known that the genetic basis of the amino acid change involves a single base difference between the normal allele DNA sequence and the mutant allele DNA sequence.

Unless combined with other techniques (such as restriction enzyme analysis), methods that allow for the same level of hybridization in the case of both partial as well as complete complementarity are typically unsuited for such applications; the probe will hybridize to both the normal and variant target sequence. Hybridization, regardless of the method used, requires some degree of complementarity between the sequence being assayed (the target sequence) and the fragment of DNA used to perform the test (the probe). Of course, those of skill in the art know that one can obtain binding without any complementarity but this binding is nonspecific and to be avoided.

As used herein, the terms "complementary" or "complementarity" are used in reference to polynucleotides (*i.e.*, a sequence of nucleotides) related by the base-pairing rules. For example, for the sequence "A-G-T," is complementary to the sequence "T-C-A." Complementarity may be "partial," in which only some of the nucleic acids' bases are matched according to the base pairing rules. Or, there may be "complete" or "total" complementarity between the nucleic acids. The degree of complementarity between nucleic acid strands has significant effects on the efficiency and strength of hybridization between nucleic acid strands. This is of particular importance in amplification reactions, as well as detection methods that depend upon binding between nucleic acids.

- 24 -

The term "homology" refers to a degree of complementarity. There may be partial homology or complete homology (*i.e.*, identity). A partially complementary sequence is one that at least partially inhibits a completely complementary sequence from hybridizing to a target nucleic acid and is referred to using the functional term "substantially homologous." The inhibition of hybridization of the completely complementary sequence to the target sequence may be examined using a hybridization assay (Southern or Northern blot, solution hybridization and the like) under conditions of low stringency. A substantially homologous sequence or probe will compete for and inhibit the binding (*i.e.*, the hybridization) of a completely homologous to a target under conditions of low stringency. This is not to say that conditions of low stringency are such that non-specific binding is permitted; low stringency conditions require that the binding of two sequences to one another be a specific (*i.e.*, selective) interaction. The absence of non-specific binding may be tested by the use of a second target which lacks even a partial degree of complementarity (*e.g.*, less than about 30% identity); in the absence of non-specific binding the probe will not hybridize to the second non-complementary target.

Numerous equivalent conditions are known in the art that may be employed to comprise low stringency conditions; factors such as the length and nature (DNA, RNA, base composition) of the probe and nature of the target (DNA, RNA, base composition, present in solution or immobilized, etc.) and the concentration of the salts and other components (*e.g.*, the presence or absence of formamide, dextran sulfate, polyethylene glycol) are considered and the hybridization solution may be varied to generate conditions of low stringency hybridization different from, but equivalent to, the above listed conditions. In addition, the art knows conditions that promote hybridization under conditions of high stringency (*e.g.*, increasing the temperature of the hybridization and/or wash steps, the use of formamide in the hybridization solution, etc.).

- 25 -

When used in reference to a double-stranded nucleic acid sequence such as a cDNA or genomic clone, the term "substantially homologous" refers to any probe which can hybridize to either or both strands of the double-stranded nucleic acid sequence under conditions of low stringency as described above.

The following terms are used to describe the sequence relationships between two or more polynucleotides: "reference sequence", "comparison window", "sequence identity", "percentage of sequence identity", and "substantial identity". The term "sequence identity" means that two polynucleotide sequences are identical (i.e., on a nucleotide-by-nucleotide basis) over the window of comparison. The term "percentage of sequence identity" is calculated by comparing two optimally aligned sequences over the window of comparison, determining the number of positions at which the identical nucleic acid base (e.g., A, T, C, G, U, or I) occurs in both sequences to yield the number of matched positions, dividing the number of matched positions by the total number of positions in the window of comparison (i.e., the window size), and multiplying the result by 100 to yield the percentage of sequence identity.

A "reference sequence" is a defined sequence used as a basis for a sequence comparison; a reference sequence may be a subset of a larger sequence, for example, as a segment of a full-length cDNA or gene sequence given in a sequence listing, such as any of the polynucleotide sequences provided herein, or may comprise a complete cDNA or gene sequence. Generally, but not always, a reference sequence is at least 20 nucleotides in length, frequently at least 25 nucleotides in length, and often at least 50 nucleotides in length. Since two polynucleotides may each (1) comprise a sequence (i.e., a portion of the complete polynucleotide sequence) that is similar between the two polynucleotides, and (2) may further comprise a sequence that is divergent between the two polynucleotides, sequence comparisons between two (or more) polynucleotides are typically performed by comparing sequences of the two polynucleotides over a "comparison

- 26 -

window" to identify and compare local regions of sequence similarity. A "comparison window", as used herein, refers to a conceptual segment of at least 20 contiguous nucleotide positions wherein a polynucleotide sequence may be compared to a reference sequence of at least 20 contiguous nucleotides and wherein the portion of the polynucleotide sequence in the comparison window may comprise additions or deletions (i.e., gaps) of 20 percent or less as compared to the reference sequence (which does not comprise additions or deletions) for optimal alignment of the two sequences. Optimal alignment of sequences for aligning a comparison window may be conducted by the local  
5  
homology algorithm of Smith and Waterman (1981) Adv. Appl. Math. 2: 482, by the homology alignment algorithm of Needleman and Wunsch (1970) J. Mol. Biol. 48: 443, by the search for similarity method of Pearson and Lipman (1988) Proc. Natl. Acad. Sci. (U.S.A.) 85: 2444, by computerized implementations of these algorithms (GAP, BESTFIT, FASTA, and TFASTA  
10  
in the Wisconsin Genetics Software Package Release 7.0, Genetics Computer Group, 575 Science Dr., Madison, Wis.), or by inspection, and the best alignment (i.e., resulting in the highest percentage of homology over the comparison window) generated by the various methods is selected. The term "substantial identity" denotes a characteristic of a polynucleotide sequence,  
15  
wherein the polynucleotide comprises a sequence that has at least 85 percent sequence identity, preferably at least 90 to 95 percent sequence identity, more usually at least 99 percent sequence identity as compared to a reference sequence over a comparison window of at least 20 nucleotide positions, frequently over a window of at least 25-50 nucleotides, wherein the percentage  
20  
of sequence identity is calculated by comparing the reference sequence to the polynucleotide sequence which may include deletions or additions which total 20 percent or less of the reference sequence over the window of comparison. The reference sequence may be a subset of a larger sequence, for example, as a segment of the full-length polynucleotide sequence or the full-length cDNA  
25  
sequence.  
30

- 27 -

As applied to polypeptides, the term "substantial identity" means that two peptide sequences, when optimally aligned, such as by the programs GAP or BESTFIT using default gap weights, share at least 80 percent sequence identity, preferably at least 90 percent sequence identity, more preferably at least 95 percent sequence identity or more (e.g., 99 percent sequence identity). Preferably, residue positions which are not identical differ by conservative amino acid substitutions. Conservative amino acid substitutions refer to the interchangeability of residues having similar side chains. For example, a group of amino acids having aliphatic side chains is glycine, alanine, valine, leucine, and isoleucine; a group of amino acids having aliphatic-hydroxyl side chains is serine and threonine; a group of amino acids having amide-containing side chains is asparagine and glutamine; a group of amino acids having aromatic side chains is phenylalanine, tyrosine, and tryptophan; a group of amino acids having basic side chains is lysine, arginine, and histidine; and a group of amino acids having sulfur-containing side chains is cysteine and methionine. Preferred conservative amino acids substitution groups are: valine-leucine-isoleucine, phenylalanine-tyrosine, lysine-arginine, alanine-valine, and asparagine-glutamine. see

A gene may produce multiple RNA species which are generated by differential splicing of the primary RNA transcript. cDNAs that are splice variants of the same gene will contain regions of sequence identity or complete homology (representing the presence of the same exon or portion of the same exon on both cDNAs) and regions of complete non-identity (for example, representing the presence of exon "A" on cDNA 1 wherein cDNA 2 contains exon "B" instead). Because the two cDNAs contain regions of sequence identity they will both hybridize to a probe derived from the entire gene or portions of the gene containing sequences found on both cDNAs; the two splice variants are therefore substantially homologous to such a probe and to each other.

When used in reference to a single-stranded nucleic acid sequence, the term "substantially homologous" refers to any probe which can hybridize (*i.e.*,



- 28 -

it is the complement of) the single-stranded nucleic acid sequence under conditions of low stringency as described. As used herein, the term "hybridization" is used in reference to the pairing of complementary nucleic acids. Hybridization and the strength of hybridization (*i.e.*, the strength of the association between the nucleic acids) is impacted by such factors as the degree of complementarity between the nucleic acids, stringency of the conditions involved, the  $T_m$  of the formed hybrid, and the G:C ratio within the nucleic acids.

The term " $T_m$ " is used in reference to the "melting temperature." The melting temperature is the temperature at which a population of double-stranded nucleic acid molecules becomes half dissociated into single strands. The equation for calculating the  $T_m$  of nucleic acids is well known in the art. As indicated by standard references, a simple estimate of the  $T_m$  value may be calculated by the equation:  $T_m = 81.5 + 0.41(\% G + C)$ , when a nucleic acid is in aqueous solution at 1 M NaCl (*See e.g.*, Anderson and Young, Quantitative Filter Hybridization, in *Nucleic Acid Hybridization* [1985]). Other references include more sophisticated computations which take structural as well as sequence characteristics into account for the calculation of  $T_m$ .

The term "stringency" is used in reference to the conditions of temperature, ionic strength, and the presence of other compounds such as organic solvents, under which nucleic acid hybridizations are conducted. With "high stringency" conditions, nucleic acid base pairing will occur only between nucleic acid fragments that have a high frequency of complementary base sequences. Thus, conditions of "weak" or "low" stringency are often required with nucleic acids that are derived from organisms that are genetically diverse, as the frequency of complementary sequences is usually less.

"Amplification" is a special case of nucleic acid replication involving template specificity. It is to be contrasted with non-specific template

- 29 -

replication (*i.e.*, replication that is template-dependent but not dependent on a specific template). Template specificity is here distinguished from fidelity of replication (*i.e.*, synthesis of the proper polynucleotide sequence) and nucleotide (ribo- or deoxyribo-) specificity. Template specificity is frequently described in terms of "target" specificity. Target sequences are "targets" in the sense that they are sought to be sorted out from other nucleic acid. Amplification techniques have been designed primarily for this sorting out.

Template specificity is achieved in most amplification techniques by the choice of enzyme. Amplification enzymes are enzymes that, under conditions they are used, will process only specific sequences of nucleic acid in a heterogeneous mixture of nucleic acid. For example, in the case of Q $\beta$  replicase, MDV-1 RNA is the specific template for the replicase (Kacian *et al.*, Proc. Natl. Acad. Sci. USA 69:3038 [1972]). Other nucleic acids will not be replicated by this amplification enzyme. Similarly, in the case of T7 RNA polymerase, this amplification enzyme has a stringent specificity for its own promoters (Chamberlin *et al.*, Nature 228:227 [1970]). In the case of T4 DNA ligase, the enzyme will not ligate the two oligonucleotides or polynucleotides, where there is a mismatch between the oligonucleotide or polynucleotide substrate and the template at the ligation junction (Wu and Wallace, Genomics 4:560 [1989]). Finally, *Taq* and *Pfu* polymerases, by virtue of their ability to function at high temperature, are found to display high specificity for the sequences bounded and thus defined by the primers; the high temperature results in thermodynamic conditions that favor primer hybridization with the target sequences and not hybridization with non-target sequences (Erich (ed.), *PCR Technology*, Stockton Press [1989]).

The term "amplifiable nucleic acid" is used in reference to nucleic acids which may be amplified by any amplification method. It is contemplated that "amplifiable nucleic acid" will usually comprise "sample template."

The term "sample template" refers to nucleic acid originating from a sample which is analyzed for the presence of "target" (defined below). In

- 30 -

contrast, "background template" is used in reference to nucleic acid other than sample template which may or may not be present in a sample. Background template is most often inadvertent. It may be the result of carryover, or it may be due to the presence of nucleic acid contaminants sought to be purified away from the sample. For example, nucleic acids from organisms other than those to be detected may be present as background in a test sample.

As used herein, the term "primer" refers to an oligonucleotide, whether occurring naturally as in a purified restriction digest or produced synthetically, which is capable of acting as a point of initiation of synthesis when placed under conditions in which synthesis of a primer extension product which is complementary to a nucleic acid strand is induced, (*i.e.*, in the presence of nucleotides and an inducing agent such as DNA polymerase and at a suitable temperature and pH). The primer is preferably single stranded for maximum efficiency in amplification, but may alternatively be double stranded. If double stranded, the primer is first treated to separate its strands before being used to prepare extension products. Preferably, the primer is an oligodeoxyribonucleotide. The primer must be sufficiently long to prime the synthesis of extension products in the presence of the inducing agent. The exact lengths of the primers will depend on many factors, including temperature, source of primer and the use of the method.

A primer is selected to be "substantially" complementary to a strand of specific sequence of the template. A primer must be sufficiently complementary to hybridize with a template strand for primer elongation to occur. A primer sequence need not reflect the exact sequence of the template. For example, a non-complementary nucleotide fragment may be attached to the 5' end of the primer, with the remainder of the primer sequence being substantially complementary to the strand. Non-complementary bases or longer sequences can be interspersed into the primer, provided that the primer sequence has sufficient complementarity with the sequence of the template to hybridize and thereby form a template primer complex for synthesis of the extension product of the primer.

- 31 -

The term "nested primers" refers to primers that anneal to the target sequence in an area that is inside the annealing boundaries used to start PCR. (See, Mullis *et al.*, *Cold Spring Harbor Symposia*, Vol. LI, pp. 263-273 [1986]). Because the nested primers anneal to the target inside the annealing boundaries of the starting primers, the predominant PCR-amplified product of the starting primers is necessarily a longer sequence, than that defined by the annealing boundaries of the nested primers. The PCR-amplified product of the nested primers is an amplified segment of the target sequence that cannot, therefore, anneal with the starting primers.

The term "probe" refers to an oligonucleotide (*i.e.*, a sequence of nucleotides), whether occurring naturally as in a purified restriction digest or produced synthetically, recombinantly or by PCR amplification, which is capable of hybridizing to another oligonucleotide of interest. A probe may be single-stranded or double-stranded. Probes are useful in the detection, identification and isolation of particular gene sequences. It is contemplated that any probe used in the present invention will be labelled with any "reporter molecule," so that is detectable in any detection system, including, but not limited to enzyme (*e.g.*, ELISA, as well as enzyme-based histochemical assays), fluorescent, radioactive, and luminescent systems. It is not intended that the present invention be limited to any particular detection system or label.

The term "label" as used herein refers to any atom or molecule which can be used to provide a detectable (preferably quantifiable) signal, and which can be attached to a nucleic acid or protein. Labels may provide signals detectable by fluorescence, radioactivity, colorimetry, gravimetry, X-ray diffraction or absorption, magnetism, enzymatic activity, and the like.

The term "target," when used in reference to the polymerase chain reaction, refers to the region of nucleic acid bounded by the primers used for polymerase chain reaction. Thus, the "target" is sought to be sorted out from other nucleic acid sequences. A "segment" is defined as a region of nucleic acid within the target sequence.

- 32 -

The term "substantially single-stranded" when used in reference to a nucleic acid target means that the target molecule exists primarily as a single strand of nucleic acid in contrast to a double-stranded target which exists as two strands of nucleic acid which are held together by inter-strand base pairing interactions.

Nucleic acids form secondary structures which depend on base-pairing for stability. When single strands of nucleic acids (single-stranded DNA, denatured double-stranded DNA or RNA) with different sequences, even closely related ones, are allowed to fold on themselves, they assume characteristic secondary structures. An alteration in the sequence of the target may cause the destruction of a duplex region(s), or an increase in stability of a thereby altering the accessibility of some regions to hybridization of the probes oligonucleotides. While not being limited to any particular theory, it is thought that individual molecules in the target population may each assume only one or a few of the structures (*i.e.*, duplexed regions), but when the sample is analyzed as a whole, a composite pattern from the hybridization of the probes can be created. Many of the structures that can alter the binding of the probes are likely to be only a few base-pairs long and would appear to be unstable. Some of these structures may be displaced by the hybridization of a probe in that region; others may be stabilized by the hybridization of a probe nearby, such that the probe/substrate duplex can stack coaxially with the target intrastrand duplex, thereby increasing the stability of both. The formation or disruption of these structures in response to small sequence changes results in changes in the patterns of probe/target complex formation. As used herein, the term "polymerase chain reaction" ("PCR") refers to the method of Mullis U.S. Patent Nos. 4,683,195 4,683,202, and 4,965,188, hereby incorporated by reference, which describe a method for increasing the concentration of a segment of a target sequence in a mixture of genomic DNA without cloning or purification. This process for amplifying the target sequence consists of introducing a large excess of two oligonucleotide primers to the DNA mixture containing the desired target sequence, followed by a precise sequence of

- 33 -

thermal cycling in the presence of a DNA polymerase. The two primers are complementary to their respective strands of the double stranded target sequence. To effect amplification, the mixture is denatured and the primers then annealed to their complementary sequences within the target molecule.

5 Following annealing, the primers are extended with a polymerase so as to form a new pair of complementary strands. The steps of denaturation, primer annealing and polymerase extension can be repeated many times (*i.e.*, denaturation, annealing and extension constitute one "cycle"; there can be numerous "cycles") to obtain a high concentration of an amplified segment of  
10 the desired target sequence. The length of the amplified segment of the desired target sequence is determined by the relative positions of the primers with respect to each other, and therefore, this length is a controllable parameter. By virtue of the repeating aspect of the process, the method is referred to as the "polymerase chain reaction" (hereinafter "PCR"). Because  
15 the desired amplified segments of the target sequence become the predominant sequences (in terms of concentration) in the mixture, they are said to be "PCR amplified".

With PCR, it is possible to amplify a single copy of a specific target sequence in genomic DNA to a level detectable by several different  
20 methodologies (*e.g.*, hybridization with a labeled probe; incorporation of biotinylated primers followed by avidin-enzyme conjugate detection; incorporation of <sup>32</sup>P-labeled deoxynucleotide triphosphates, such as dCTP or dATP, into the amplified segment). In addition to genomic DNA, any oligonucleotide or polynucleotide sequence can be amplified with the  
25 appropriate set of primer molecules. In particular, the amplified segments created by the PCR process itself are, themselves, efficient templates for subsequent PCR amplifications.

The terms "PCR product," "PCR fragment," and "amplification product" refer to the resultant mixture of compounds after two or more cycles  
30 of the PCR steps of denaturation, annealing and extension are complete.

- 34 -

These terms encompass the case where there has been amplification of one or more segments of one or more target sequences.

The term "amplification reagents" refers to those reagents (deoxyribonucleotide triphosphates, buffer, etc.), needed for amplification except for primers, nucleic acid template and the amplification enzyme. Typically, amplification reagents along with other reaction components are placed and contained in a reaction vessel (test tube, microwell, etc.).

As used in reference to amplification methods such as PCR, the term "polymerase" refers to any polymerase suitable for use in the amplification of nucleic acids of interest. It is intended that the term encompass such DNA polymerases as the polymerase III of the present invention, as well as *Taq* DNA polymerase (*i.e.*, the type I polymerase obtained from *Thermus aquaticus*), although other polymerases, both thermostable and thermolabile are also encompassed by this definition.

The term "RT-PCR" refers to the replication and amplification of RNA sequences. In this method, reverse transcription is coupled to PCR, most often using a one enzyme procedure in which a thermostable polymerase is employed, as described in U.S. Patent No. 5,322,770, herein incorporated by reference. In RT-PCR, the RNA template is converted to cDNA due to the reverse transcriptase activity of the polymerase, and then amplified using the polymerizing activity of the polymerase (*i.e.*, as in other PCR methods). The proteins and polypeptides of the invention can be used in any method of synthesizing or replicating DNA.

The terms "restriction endonucleases" and "restriction enzymes" refer to bacterial enzymes, each of which cut double-stranded DNA at or near a specific nucleotide sequence.

The term "recombinant DNA molecule" as used herein refers to a DNA molecule which is comprised of segments of DNA joined together by means of molecular biological techniques.

The terms "in operable combination," "in operable order," and "operably linked" refer to the linkage of nucleic acid sequences in such a

- 35 -

manner that a nucleic acid molecule capable of directing the transcription of a given gene and/or the synthesis of a desired protein molecule is produced. The term also refers to the linkage of amino acid sequences in such a manner so that a functional protein is produced.

5           As used herein, the term "isolated" when used in relation to a nucleic acid, as in "an isolated oligonucleotide" or "isolated polynucleotide" refers to a nucleic acid sequence that is identified and separated from at least one contaminant nucleic acid with which it is ordinarily associated in its natural source. Isolated nucleic acid is such present in a form or setting that is  
10           different from that in which it is found in nature. In contrast, non-isolated nucleic acids as nucleic acids such as DNA and RNA found in the state they exist in nature. The isolated nucleic acid, oligonucleotide, or polynucleotide may be present in single-stranded or double-stranded form. When an isolated nucleic acid, oligonucleotide or polynucleotide is to be utilized to express a  
15           protein, the oligonucleotide or polynucleotide will contain at a minimum the sense or coding strand (*i.e.*, the oligonucleotide or polynucleotide may single-stranded), but may contain both the sense and anti-sense strands (*i.e.*, the oligonucleotide or polynucleotide may be double-stranded).

          As used herein, the term "purified" or "to purify" refers to the removal  
20           of contaminants from a sample. For example, anti-DNA polymerase III holoenzyme and holoenzyme subunit and accessory protein antibodies are purified by removal of contaminating non-immunoglobulin proteins; they are also purified by the removal of immunoglobulin that does not bind DNA polymerase III holoenzyme or holoenzyme subunit or accessory proteins. The  
25           removal of non-immunoglobulin proteins and/or the removal of immunoglobulins that do not bind DNA polymerase III holoenzyme or holoenzyme subunit or accessory proteins results in an increase in the percent of DNA polymerase III holoenzyme or holoenzyme subunit or accessory protein-reactive immunoglobulins in the sample. In another example,  
30           recombinant DNA polymerase III holoenzyme or holoenzyme subunit or accessory protein polypeptides are expressed in bacterial host cells and the



- 36 -

polypeptides are purified by the removal of host cell proteins; the percent of recombinant DNA polymerase III holoenzyme or holoenzyme subunit or accessory protein polypeptides is thereby increased in the sample.

5 The term "recombinant DNA molecule" as used herein refers to a DNA molecule which is comprised of segments of DNA joined together by means of molecular biological techniques.

The term "recombinant protein" or "recombinant polypeptide" as used herein refers to a protein molecule which is expressed from a recombinant DNA molecule.

10 The term "native protein" as used herein to indicate that a protein does not contain amino acid residues encoded by vector sequences; that is the native protein contains only those amino acids found in the protein as it occurs in nature. A native protein may be produced by recombinant means or may be isolated from a naturally occurring source.

15 As used herein, the term "fusion protein" refers to a chimeric protein containing the protein of interest (*i.e.*, DNA polymerase III holoenzyme or holoenzyme subunit or accessory proteins and fragments of the holoenzyme, subunit or accessory protein) joined to a fusion partner, which is an exogenous protein or peptide fragment. The fusion partner consists of a non-DNA  
20 polymerase III holoenzyme or holoenzyme subunit protein or accessory protein. The fusion partner may enhance solubility of the DNA polymerase III holoenzyme or holoenzyme subunit protein or accessory protein as expressed in a host cell, may provide an affinity tag to allow purification of the recombinant fusion protein from the host cell or culture supernatant, or both.  
25 If desired, the fusion protein may be removed from the protein of interest (*i.e.*, DNA polymerase III holoenzyme, holoenzyme subunit protein, or accessory proteins or fragments of any of the foregoing) by a variety of enzymatic or chemical means known to the art.

30 In the present invention, the subunits and accessory proteins of the invention are fused to an N-terminal peptide that contains a hexahistidine site, a biotinylation site and a thrombin cleavage site. In other embodiments, the

- 37 -

subunits and accessory proteins are expressed as translationally coupled proteins. In yet another embodiment, the amino terminal tag comprises a hexahistidine site and a biotinylation site. In yet another embodiment, the subunits and accessory proteins of the invention are fused to a C-terminal peptide comprising a hexahistidine site and a biotinylation site. Other marker sequences are known in the art and can be linked to the subunits and accessory proteins of the invention.

A "variant" of DNA polymerase III holoenzyme or holoenzyme subunit or accessory protein refers to an amino acid sequence that is altered by one or more amino acids. The variant may have "conservative" changes, wherein a substituted amino acid has similar structural or chemical properties (*e.g.*, replacement of leucine with isoleucine). More rarely, a variant may have "nonconservative" changes (*e.g.*, replacement of a glycine with a tryptophan). Similar minor variations may also include amino acid deletions or insertions, or both. Guidance in determining which amino acid residues may be substituted, inserted, or deleted without abolishing biological or immunological activity may be found using computer programs well known in the art, for example, DNASTAR software.

The term "sequence variation" refers to differences in nucleic acid sequence between two nucleic acid templates. For example, a wild-type structural gene and a mutant form of this wild-type structural gene may vary in sequence by the presence of single base substitutions and/or deletions or insertions of one or more nucleotides. These two forms of the structural gene are said to vary in sequence from one another. A second mutant form of the structural gene may exist. This second mutant form is said to vary in sequence from both the wild-type gene and the first mutant form of the gene. It is noted, however, that the invention does not require that a comparison be made between one or more forms of a gene to detect sequence variations. Because the method of the invention generates a characteristic and reproducible pattern of complex formation for a given nucleic acid target, a characteristic "fingerprint" may be obtained from any nucleic target without reference to a

- 38 -

wild-type or other control. The invention contemplates the use of the method for both "fingerprinting" nucleic acids without reference to a control and identification of mutant forms of a target nucleic acid by comparison of the mutant form of the target with a wild-type or known mutant control.

5           The term "target nucleic acid" refers to the region of nucleic acid bounded by the primers used for polymerase chain reaction. Thus, the "target" is sought to be sorted out from other nucleic acid sequences. A "segment" is defined as a region of nucleic acid within the target sequence.

10           The term "nucleotide analog" refers to modified or non-naturally occurring nucleotides such as 7-deaza purines (*i.e.*, 7-deaza-dATP and 7-deaza-dGTP). Nucleotide analogs include base analogs and comprise modified forms of deoxyribonucleotides as well as ribonucleotides. The term "nucleotide analog" when used in reference to targets present in a PCR mixture refers to the use of nucleotides other than dATP, dGTP, dCTP and  
15           dTTP; thus, the use of dUTP (a naturally occurring dNTP) in a PCR would comprise the use of a nucleotide analog in the PCR. A PCR product generated using dUTP, 7-deaza-dATP, 7-deaza-dGTP or any other nucleotide analog in the reaction mixture is said to contain nucleotide analogs.

20           Oligonucleotide primers matching or complementary to a gene sequence refers to oligonucleotide primers capable of facilitating the template-dependent synthesis of single or double-stranded nucleic acids. Oligonucleotide primers matching or complementary to a gene sequence may be used in PCRs, RT-PCRs and the like.

25           A "consensus gene sequence" refers to a gene sequence which is derived by comparison of two or more gene sequences and which describes the nucleotides most often present in a given segment of the genes; the consensus sequence is the canonical sequence. "Consensus protein," "consensus amino acid," consensus peptide," and consensus polypeptide sequences refer to sequences that are shared between multiple organisms or  
30           proteins.

- 39 -

The term "biologically active," refers to a protein or other biologically active molecules (*e.g.*, catalytic RNA) having structural, regulatory, or biochemical functions of a naturally occurring molecule. Likewise, "immunologically active" refers to the capability of the natural, recombinant, or synthetic DNA polymerase III holoenzyme or holoenzyme subunit, or accessory proteins, or any oligopeptide or polynucleotide thereof, to induce a specific immune response in appropriate animals or cells and to bind with specific antibodies.

The term "agonist" refers to a molecule which, when bound to DNA polymerase III holoenzyme or holoenzyme subunit or accessory protein, causes a change in DNA polymerase III holoenzyme or holoenzyme subunit or accessory protein, which modulates the activity of DNA polymerase III holoenzyme or holoenzyme subunit or accessory protein. Agonists may include proteins, nucleic acids, carbohydrates, or any other molecules which bind or interact with DNA polymerase III holoenzyme or holoenzyme subunit or accessory protein.

The terms "antagonist" or "inhibitor" refer to a molecule which, when bound to DNA polymerase III holoenzyme or holoenzyme subunit, blocks or modulates the biological or immunological activity of DNA polymerase III holoenzyme or holoenzyme subunit or accessory protein. Antagonists and inhibitors may include proteins, nucleic acids, carbohydrates, or any other molecules which bind or interact with DNA polymerase III holoenzyme or holoenzyme subunit or accessory protein.

The term "modulate" refers to a change or an alteration in the biological activity of DNA polymerase III holoenzyme or holoenzyme subunit or accessory protein. Modulation may be an increase or a decrease in protein activity, a change in binding characteristics, or any other change in the biological, functional, or immunological properties of DNA polymerase III holoenzyme or holoenzyme subunit or accessory protein.

The term "derivative" refers to the chemical modification of a nucleic acid encoding DNA polymerase III holoenzyme or holoenzyme subunit or

- 40 -

accessory protein, or the encoded DNA polymerase III holoenzyme or holoenzyme subunit or accessory protein. Illustrative of such modifications would be replacement of hydrogen by an alkyl, acyl, or amino group. A nucleic acid derivative would encode a polypeptide which retains essential biological characteristics of the natural molecule.

The term "Southern blot (analysis)" refers to the analysis of DNA on agarose or acrylamide gels to fractionate the DNA according to size followed by transfer of the DNA from the gel to a solid support, such as nitrocellulose or a nylon membrane. The immobilized DNA is then probed with a labeled probe to detect DNA species complementary to the probe used. The DNA may be cleaved with restriction enzymes prior to electrophoresis. Following electrophoresis, the DNA may be partially depurinated and denatured prior to or during transfer to the solid support. Southern blots are a standard tool of molecular biologists (Sambrook *et al.*, *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Press, NY, pp 9.31-9.58 [1989]).

The term "Northern blot (analysis)" refers to the analysis of RNA by electrophoresis of RNA on agarose gels to fractionate the RNA according to size followed by transfer of the RNA from the gel to a solid support, such as nitrocellulose or a nylon membrane. The immobilized RNA is then probed with a labeled probe to detect RNA species complementary to the probe used. Northern blots are a standard tool of molecular biologists (Sambrook *et al.*, *supra*, pp 7.39-7.52 [1989]).

The term "Western blot" or "Western analysis" refers to the analysis of protein(s) (or polypeptides) immobilized onto a support such as nitrocellulose or a membrane. The proteins are run on acrylamide gels to separate the proteins, followed by transfer of the protein from the gel to a solid support, such as nitrocellulose or a nylon membrane. The immobilized proteins are then exposed to antibodies with reactivity against an antigen of interest. The binding of the antibodies may be detected by various methods, including the use of radiolabelled antibodies.

- 41 -

An "immunogenic epitope" is defined as a part of a protein that elicits an antibody response when the whole protein is the immunogen. These immunogenic epitopes are believed to be confined to a few loci on the molecule. On the other hand, a region of a protein molecule to which an antibody can bind is defined as an "antigenic epitope." The number of immunogenic epitopes of a protein is generally less than the number of antigenic epitopes. See, for instance, Geysen, et al., Proc. Natl. Acad. Sci. USA 81:3998-4002 (1983). See, for example, USPN 6,011,012.

The term "antigenic determinant" as used herein refers to that portion of an antigen that makes contact with a particular antibody (*i.e.*, an epitope). When a protein or fragment of a protein is used to immunize a host animal, numerous regions of the protein may induce the production of antibodies which bind specifically to a given region or three-dimensional structure on the protein; these regions or structures are referred to as antigenic determinants. An antigenic determinant may compete with the intact antigen (*i.e.*, the "immunogen" used to elicit the immune response) for binding to an antibody. See, for example, USPN 6,011,012.

The terms "specific binding" or "specifically binding" when used in reference to the interaction of an antibody and a protein or peptide means that the interaction is dependent upon the presence of a particular structure (*i.e.*, the antigenic determinant or epitope) on the protein; in other words the antibody is recognizing and binding to a specific protein structure rather than to proteins in general. For example, if an antibody is specific for epitope "A," the presence of a protein containing epitope A (or free, unlabelled A) in a reaction containing labelled "A" and the antibody will reduce the amount of labelled A bound to the antibody.

As used herein, the term "cell culture" refers to any *in vitro* culture of cells. Included within this term are continuous cell lines (*e.g.*, with an immortal phenotype), primary cell cultures, finite cell lines (*e.g.*, non-transformed cells), and any other cell population maintained *in vitro*.

- 42 -

The terms "test DNA polymerase III holoenzyme" and "test holoenzyme subunit" or "test protein" refers to a sample suspected of containing DNA polymerase III holoenzyme or holoenzyme subunit or accessory protein, respectively. The concentration of DNA polymerase III holoenzyme or holoenzyme subunit or accessory protein in the test sample is determined by various means, and may be compared with a "quantitated amount of DNA polymerase III holoenzyme or holoenzyme subunit or accessory protein" (*i.e.*, a positive control sample containing a known amount of DNA polymerase III holoenzyme or holoenzyme subunit or accessory protein), in order to determine whether the concentration of test DNA polymerase III holoenzyme or holoenzyme subunit or accessory protein in the sample is within the range usually found within samples from wild-type organisms.

The term "microorganism" or "organism" as used herein means an organism too small to be observed with the unaided eye and includes, but is not limited to bacteria, virus, protozoans, fungi, and ciliates.

The term "microbial gene sequences" refers to gene sequences derived from a microorganism.

The term "bacteria" refers to any bacterial species including eubacterial and archaeobacterial species.

The term "virus" refers to obligate, ultramicroscopic, intracellular parasites incapable of autonomous replication (*i.e.*, replication requires the use of the host cell's machinery).

## B. Methodologies

Unless otherwise indicated, all nucleotide sequences determined by sequencing a DNA molecule herein were determined using an automated DNA sequencer. A variety of sequencers are known in the art, such as the Model 373 from Applied Biosystems, Inc., for example. Amino acid sequences of polypeptides encoded by DNA molecules determined herein

- 43 -

were predicted by translation of a DNA sequence determined as above. Alternatively the sequence can be determined by directly sequencing the polypeptide. As is known in the art for any DNA sequence determined by this automated approach, any nucleotide sequence determined herein may contain  
5 some errors. Nucleotide sequences determined by automation are typically at least about 90% identical, more typically at least about 95% to at least about 99.9% identical to the actual nucleotide sequence of the sequenced DNA molecule. The actual sequence can be more precisely determined by other approaches including manual DNA sequencing methods well known in the art.  
10 As is also known in the art, a single insertion or deletion in a determined nucleotide sequence cause a frame shift in translation of the nucleotide sequence such that the predicted amino acid sequence compared to the actual sequence will encoded by a determined nucleotide sequence will be completely different from the amino acid sequence actually encoded by the  
15 sequenced DNA molecule, beginning at the point of such an insertion or deletion. See for example, USPN 6,171,816 and 6,040,157.

"Identity" per se has an art-recognized meaning and can be calculated using published techniques. (See, e.g.: (COMPUTATIONAL MOLECULAR BIOLOGY, Lesk, A. M., ed., Oxford University Press, New York, (1988);  
20 BIOCOMPUTING: INFORMATICS AND GENOME PROJECTS, Smith, D. W., ed., Academic Press, New York, (1993); COMPUTER ANALYSIS OF SEQUENCE DATA, PART I, Griffin, A. M., and Griffin, H. G., eds., Humana Press, New Jersey, (1994); SEQUENCE ANALYSIS IN MOLECULAR BIOLOGY, von Heinje, G., Academic Press, (1987); and  
25 SEQUENCE ANALYSIS PRIMER, Gribskov, M. and Devereux, J., eds., M Stockton Press, New York, (1991).) While there exists a number of methods to measure identity between two polynucleotide or polypeptide sequences, the term "identity" is well known to skilled artisans. (Carillo, H., and Lipton, D., SIAM J Applied Math 48:1073 (1988)). Methods commonly employed to  
30 determine identity or similarity between two sequences include, but are not limited to, those disclosed in Guide to Huge Computers, Martin J. Bishop, ed.,



- 44 -

Academic Press, San Diego, (1994), and Carillo, H., and Lipton, D., SIAM J Applied Math 48:1073 (1988). Methods for aligning polynucleotides or polypeptides are codified in computer programs, including the GCG program package (Devereux, J., et al., Nucleic Acids Research (1984) 12(1):387 (1984)), BLASTP, BLASTN, FASTA (Atschul, S. F. et al., J. Molec. Biol. 215:403 (1990), Bestfit program (Wisconsin Sequence Analysis Package, Version 8 for Unix, Genetics Computer Group, University Research Park, 575 Science Drive, Madison, Wis. 53711 (using the local homology algorithm of Smith and Waterman, Advances in Applied Mathematics 2:482-489 (1981))).  
See USPN 6,040,157

In certain embodiments, polynucleotides of the invention comprise a nucleic acid, the sequence of which is at least 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98% or 99% identical to a sequence selected from the group consisting of SEQ ID NOS: 9, 16, 22, 31, 36, 67, 71, 75 and 81, or a complementary sequence thereof.

By a polynucleotide comprising a nucleic acid, the sequence of which is at least, for example, 95% "identical" to a reference nucleotide sequence is intended that the nucleic acid sequence is identical to the reference sequence except that the nucleic acid sequence may include up to five point mutations per each 100 nucleotides of the reference nucleic acid sequence. In other words, to obtain a nucleic acid, the sequence of which is at least 95% identical to a reference nucleic acid sequence, up to 5% of the nucleotides in the reference sequence may be deleted or substituted with another nucleotide, or a number of nucleotides up to 5% of the total nucleotides in the reference sequence may be inserted into the reference sequence. These mutations of the reference sequence may occur at the 5' or 3' terminal positions of the reference nucleotide sequence or anywhere between those terminal positions, interspersed either individually among nucleotides in the reference sequence or in one or more contiguous groups within the reference sequence. The reference (query) sequence may be any one of the entire nucleotide sequences shown in SEQ ID NOS: 9, 16, 22, 31, 36, 67, 71, 75 and 81, or any fragment

- 45 -

of any of these sequences, as described *infra*. See USPN 6,040,157 and 6,171,816, for example.

As a practical matter, whether any particular nucleic acid molecule is at least 95%, 96%, 97%, 98% or 99% identical to, for instance, the nucleotide sequence shown in SEQ ID NOS: 9, 16, 22, 31, 36, 67, 71, 75 and 81, can be determined conventionally using known computer programs such as the Bestfit program (Wisconsin Sequence Analysis Package, Version 8 for Unix, Genetics Computer Group, University Research Park, 575 Science Drive, Madison, Wis. 53711). Bestfit uses the local homology algorithm of Smith and Waterman, *Advances in Applied Mathematics* 2:482-489 (1981), to find the best segment of homology between two sequences. When using Bestfit or any other sequence alignment program to determine whether a particular sequence is, for instance, 95% identical to a reference sequence according to the present invention, the parameters are set, of course, such that the percentage of identity is calculated over the full length of the reference nucleotide sequence and that gaps in homology of up to 5% of the total number of nucleotides in the reference sequence are allowed. Other sequence analysis programs, known in the art, can be used to determine percent identity. See USPN 6,040,157 and 6,171,816.

Of course, due to the degeneracy of the genetic code, one of ordinary skill in the art will immediately recognize that a large number of the nucleic acid molecules having a sequence at least 95%, 96%, 97%, 98%, or 99% identical to the nucleic acid sequences shown in SEQ ID NOS: 9, 16, 22, 31, 36, 67, 71, 75 and 81 will encode a polypeptide or protein having biological activity. In fact, since degenerate variants of these nucleotide sequences all encode the same polypeptide, this will be clear to the skilled artisan even without performing the comparison assays. It will be further recognized in the art that, for such nucleic acid molecules that are not degenerate variants, a reasonable number will also encode a polypeptide have biological activity. This is because the skilled artisan is fully aware of amino acid substitutions that are either less likely or not likely to significantly effect protein function

- 46 -

(e.g., replacing one aliphatic amino acid with a second aliphatic amino acid).  
See, USPN 6,011,012; 6,171,186; 6,040,157.

One embodiment of the present invention is directed to polynucleotides comprising a nucleic acid, the sequence of which is at least  
5 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98% or 99% identical to a nucleic acid sequence of SEQ ID NOS: 9, 16, 22, 31, 36, 67, 71, 75 and 81, or a complementary sequence thereof, irrespective of whether they have functional activity. This is because even where a particular polynucleotide does not have functional activity, one of skill in the art would still know how  
10 to use the nucleic acid molecule, for instance, as a hybridization probe, an S1 nuclease mapping probe, or a polymerase chain reaction (PCR) primer.

Preferred, however, are polynucleotides comprising a nucleic acid, the sequence of which is at least 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%,  
15 98% or 99% identical to a nucleic acid sequence of SEQ ID NOS: 9, 16, 22, 31, 36, 67, 71, 75 and 81, or a complementary sequence thereof, which do, in fact, encode proteins which have functional activity.

The present invention further relates to variants of the nucleic acid molecules of the present invention, which encode portions, analogs or derivatives of the DNA III subunits and accessory proteins. Variants may  
20 occur naturally, such as a natural allelic variant. By an "allelic variant" is intended one of several alternate forms of a gene occupying a given locus on a chromosome of an organism. Genes II, Lewin, B., ed., John Wiley & Sons, New York (1985).

Non-naturally occurring variants may be produced using art-known  
25 mutagenesis techniques. Such variants include those produced by nucleotide substitutions, deletions or additions. The substitutions, deletions or additions may involve one or more nucleotides. The variants may be altered in coding regions, non-coding regions, or both. Alterations in the coding regions may produce conservative or non-conservative amino acid substitutions, deletions  
30 or additions. Especially preferred among these are silent substitutions, additions and deletions, which do not alter the properties and activities of the

- 47 -

DNA Pol III subunits and accessory proteins or fragments or portions thereof. Also especially preferred in this regard are conservative substitutions. Most highly preferred are nucleic acid molecules encoding the mature proteins having the amino acid sequence shown in SEQ ID NOS: 10, 17, 23, 32, 37, 68, 72, 76 and 82.

By a polypeptide having an amino acid sequence at least, for example, 95% "identical" to a reference amino acid sequence of a polypeptide is intended that the amino acid sequence of the claimed polypeptide is identical to the reference sequence except that the claimed polypeptide sequence may include up to five amino acid alterations per each 100 amino acids of the reference amino acid of the polypeptide. In other words, to obtain a polypeptide having an amino acid sequence at least 95% identical to a reference amino acid sequence, up to 5% of the amino acid residues in the reference sequence may be deleted or substituted with another amino acid, or a number of amino acids up to 5% of the total amino acid residues in the reference sequence may be inserted into the reference sequence. These alterations of the reference sequence may occur at the amino or carboxy terminal positions of the reference amino acid sequence or anywhere between those terminal positions, interspersed either individually among residues in the reference sequence or in one or more contiguous groups within the reference sequence.

As a practical matter, whether any particular polypeptide is at least 80%, 85%, 90%, 92%, 95%, 96%, 97%, 98% or 99% identical to, for instance, the amino acid sequence shown in SEQ ID NOS: 10, 17, 23, 32, 37, 68, 72, 76 and 82 or to the amino acid sequence encoded by a nucleic acid sequence can be determined conventionally using known computer programs such the Bestfit program (Wisconsin Sequence Analysis Package, Version 8 for Unix, Genetics Computer Group, University Research Park, 575 Science Drive, Madison, WI 53711). When using Bestfit or any other sequence alignment program to determine whether a particular sequence is, for instance, 95% identical to a reference sequence according to the present invention, the

- 48 -

parameters are set, of course, such that the percentage of identity is calculated over the full length of the reference amino acid sequence and that gaps in homology of up to 5% of the total number of amino acid residues in the reference sequence are allowed. See for example, USPN 6,040,157 and  
5 6,171,816.

For example, the identity between a reference sequence (query sequence, a sequence of the present invention) and a subject sequence, also referred to as a global sequence alignment, is determined using the FASTDB computer program based on the algorithm of Brutlag *et al.* (Comp. App. Biosci. 6:237-245 (1990)). Preferred parameters used in a FASTDB amino  
10 acid alignment are: Matrix=PAM 0, k-tuple=2, Mismatch Penalty=1, Joining Penalty=20, Randomization Group Length=0, Cutoff Score=1, Window Size=sequence length, Gap Penalty=5, Gap Size Penalty=0.05, Window Size=500 or the length of the subject amino acid sequence, whichever is shorter. According to this embodiment, if the subject sequence is shorter than  
15 the query sequence due to N- or C-terminal deletions, not because of internal deletions, a manual correction is made to the results to take into consideration the fact that the FASTDB program does not account for N- and C-terminal truncations of the subject sequence when calculating global percent identity.  
20 For subject sequences truncated at the N- and C-termini, relative to the query sequence, the percent identity is corrected by calculating the number of residues of the query sequence that are N- and C-terminal of the subject sequence, which are not matched/aligned with a corresponding subject residue, as a percent of the total bases of the query sequence. A determination  
25 of whether a residue is matched/aligned is determined by results of the FASTDB sequence alignment. This percentage is then subtracted from the percent identity, calculated by the above FASTDB program using the specified parameters, to arrive at a final percent identity score. This final percent identity score is what is used for the purposes of this embodiment.  
30 Only residues to the N- and C-termini of the subject sequence, which are not matched/aligned with the query sequence, are considered for the purposes of

- 49 -

manually adjusting the percent identity score. That is, only query residue positions outside the farthest N- and C-terminal residues of the subject sequence. For example, a 90 amino acid residue subject sequence is aligned with a 100 residue query sequence to determine percent identity. The deletion occurs at the N-terminus of the subject sequence and therefore, the FASTDB alignment does not show a matching/alignment of the first 10 residues at the N-terminus. The 10 unpaired residues represent 10% of the sequence (number of residues at the N- and C-termini not matched/total number of residues in the query sequence) so 10% is subtracted from the percent identity score calculated by the FASTDB program. If the remaining 90 residues were perfectly matched the final percent identity would be 90%. In another example, a 90 residue subject sequence is compared with a 100 residue query sequence. This time the deletions are internal deletions so there are no residues at the N- or C-termini of the subject sequence which are not matched/aligned with the query. In this case the percent identity calculated by FASTDB is not manually corrected. Once again, only residue positions outside the N- and C-terminal ends of the subject sequence, as displayed in the FASTDB alignment, which are not matched/aligned with the query sequence are manually corrected for. See for example, USPN 6,040,157.

Guidance concerning how to make phenotypically silent amino acid substitutions is provided in Bowie, J. U. et al., "Deciphering the Message in Protein Sequences: Tolerance to Amino Acid Substitutions," *Science* 247:1306-1310 (1990), wherein the authors indicate that there are two main approaches for studying the tolerance of an amino acid sequence to change. The first method relies on the process of evolution, in which mutations are either accepted or rejected by natural selection. The second approach uses genetic engineering to introduce amino acid changes at specific positions of a cloned gene and selections or screens to identify sequences that maintain functionality. As the authors state, these studies have revealed that proteins are surprisingly tolerant of amino acid substitutions. The authors further indicate which amino acid changes are likely to be permissive at a certain position of

- 50 -

the protein. For example, most buried amino acid residues require nonpolar side chains, whereas few features of surface side chains are generally conserved. Other such phenotypically silent substitutions are described in Bowie, J. U. et al., supra, and the references cited therein. See for example,  
5      USPN 6,040,157 and 6,171,816.

The DNA Pol III subunit polypeptides and accessory proteins of the invention may be expressed in a modified form, such as a fragment or a fusion protein, and may include not only secretion signals, but also additional heterologous functional regions. For instance, a region of additional amino  
10     acids, particularly charged amino acids, may be added to the N-terminus of the polypeptide to improve stability and persistence in the host cell, during purification, or during subsequent handling and storage. Alternatively, a region of amino acids may be added to the C-terminus of the polypeptide. Methods for adding N-terminal linked peptides and C-terminal linked peptides  
15     are known in the art. Also, peptide moieties may be added to the polypeptide to facilitate purification. Such regions may be removed prior to final preparation of the polypeptide. The addition of peptide moieties to polypeptides to engender secretion or excretion, to improve stability and to facilitate purification, among others, are familiar and routine techniques in the  
20     art. Many such peptide moieties are known in the art and contemplated for use in the practice of the invention herei.

The present invention also provides methods for producing anti-DNA polymerase III holoenzyme and anti-DNA polymerase III holoenzyme subunit and anti accessory protein antibodies comprising, exposing an animal having  
25     immunocompetent cells to an immunogen comprising at least an antigenic portion (determinant) of DNA polymerase III holoenzyme (or holoenzyme subunit or accessory) protein, under conditions such that immunocompetent cells produce antibodies directed against the portion of DNA polymerase III protein holoenzyme or holoenzyme subunit or accessory protein. In one  
30     embodiment, the method further comprises the step of harvesting the antibodies. In an alternative embodiment, the method comprises the step of

- 51 -

fusing the immunocompetent cells with an immortal cell line under conditions such that a hybridoma is produced.

The antibodies used in the methods invention may be prepared using various immunogens. In one embodiment, the immunogen is DNA  
5 polymerase III holoenzyme or holoenzyme subunit peptide, to generate antibodies that recognize DNA polymerase III holoenzyme or holoenzyme subunit(s). Antibodies binding to accessory proteins are prepared using identical or similar methods. Such antibodies include, but are not limited to polyclonal, monoclonal, chimeric, single chain, Fab fragments, and an Fab  
10 expression library.

Various procedures known in the art may be used for the production of polyclonal antibodies to DNA polymerase III holoenzyme or holoenzyme subunit or accessory proteins. For the production of antibody, various host animals can be immunized by injection with the peptide corresponding to the  
15 DNA polymerase III holoenzyme or holoenzyme subunit or accessory protein epitope including but not limited to rabbits, mice, rats, sheep, goats, etc. In a preferred embodiment, the peptide is conjugated to an immunogenic carrier (*e.g.*, diphtheria toxoid, bovine serum albumin (BSA), or keyhole limpet hemocyanin [KLH]). Various adjuvants may be used to increase the  
20 immunological response, depending on the host species, including but not limited to Freund's (complete and incomplete), mineral gels such as aluminum hydroxide, surface active substances such as lysolecithin, pluronic polyols, polyanions, peptides, oil emulsions, keyhole limpet hemocyanins, and dinitrophenol.

For preparation of monoclonal antibodies directed toward DNA  
25 polymerase III holoenzyme or holoenzyme subunit or accessory protein, any technique that provides for the production of antibody molecules by continuous cell lines in culture may be used (*See, e.g.*, Harlow and Lane, *Antibodies: A Laboratory Manual*, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY). These include but are not limited to the hybridoma  
30



- 52 -

technique originally developed by Köhler and Milstein (Köhler and Milstein, Nature 256:495-497 [1975]), as well as other techniques known in the art.

According to the invention, techniques described for the production of single chain antibodies (U.S. Patent 4,946,778; herein incorporated by reference) can be adapted to produce DNA polymerase III holoenzyme or holoenzyme subunit or accessory protein -specific single chain antibodies. An additional embodiment of the invention utilizes the techniques described for the construction of Fab expression libraries (Huse *et al.*, Science 246:1275-1281 [1989]) to allow rapid and easy identification of monoclonal Fab fragments with the desired specificity for DNA polymerase III holoenzyme or holoenzyme subunit or accessory proteins.

Antibody fragments which contain the idiotype (antigen binding region) of the antibody molecule can be generated by known techniques. For example, such fragments include but are not limited to: the F(ab')<sub>2</sub> fragment which can be produced by pepsin digestion of the antibody molecule; the Fab' fragments which can be generated by reducing the disulfide bridges of the F(ab')<sub>2</sub> fragment, and the Fab fragments which can be generated by treating the antibody molecule with papain and a reducing agent.

In the production of antibodies, screening for the desired antibody can be accomplished by techniques known in the art (*e.g.*, radioimmunoassay, ELISA [enzyme-linked immunosorbent assay], "sandwich" immunoassays, immunoradiometric assays, gel diffusion precipitin reactions, immunodiffusion assays, *in situ* immunoassays [using colloidal gold, enzyme or radioisotope labels, for example], Western Blots, precipitation reactions, agglutination assays (*e.g.*, gel agglutination assays, hemagglutination assays, etc.), complement fixation assays, immunofluorescence assays, protein A assays, and immunoelectrophoresis assays, etc.

In one method, antibody binding is detected by detecting a label on the primary antibody. In another method, the primary antibody is detected by detecting binding of a secondary antibody or reagent to the primary antibody. In a further method, the secondary antibody is labeled. Many means are

- 53 -

known in the art for detecting binding in an immunoassay and are within the scope of the present invention. (As is well known in the art, the immunogenic peptide should be provided free of the carrier molecule used in any immunization protocol. For example, if the peptide was conjugated to KLH, it may be conjugated to BSA, or used directly, in a screening assay.)

The foregoing antibodies can be used in methods known in the art relating to the localization and structure of DNA polymerase III holoenzyme or holoenzyme subunit or accessory protein (*e.g.*, for Western blotting), measuring levels thereof in appropriate biological samples, etc. The biological samples can be tested directly for the presence of DNA polymerase III holoenzyme or holoenzyme subunit or accessory protein using an appropriate strategy (*e.g.*, ELISA or radioimmunoassay) and format (*e.g.*, microwells, dipstick [*e.g.*, as described in International Patent Publication WO 93/03367], etc.). Alternatively, proteins in the sample can be size separated (*e.g.*, by polyacrylamide gel electrophoresis (PAGE), in the presence or not of sodium dodecyl sulfate (SDS), and the presence of DNA polymerase III holoenzyme or holoenzyme subunit detected by immunoblotting (Western blotting). Immunoblotting techniques are generally more effective with antibodies generated against a peptide corresponding to an epitope or antigenic determinant of a protein, and hence, are particularly suited to the present invention.

The present invention provides isolated DNA polymerase III holoenzyme subunits and accessory proteins from a thermophilic organism. In preferred embodiments, the thermophilic organism is a thermophilic organism. The thermophilic organism can be selected from a member of the genera *Thermus*, *Thermotoga*, and *Aquifex*.

The present invention also provides full-length polypeptides or proteins. The invention also provides methods for providing, as well, fragments of any size of the protein (*i.e.*, the entire amino acid sequence of the protein, as well as short peptides). Primers and gene amplification techniques are used to amplify the nucleotide sequence encoding the nucleotide region of

- 54 -

interest, which upon ligation into a vector and transfection into a host cell, results in expression of the protein or peptide of interest.

The invention is directed to an isolated polypeptide wherein said polypeptide comprises an amino acid sequence having at least 95% sequence identity to the amino acid sequence of SEQ ID NO: (uvrD helicase) 68. In one embodiment, the polypeptide has the amino acid sequence of SEQ ID NO: 68. In another embodiment, the invention is directed to an isolated polynucleotide molecule comprising a nucleotide sequence encoding a polypeptide comprising an amino acid sequence having at least 95% sequence identity to the amino acid sequence of SEQ ID NO: (uvrD helicase) 68. In a different embodiment, the isolated polynucleotide molecule comprises a nucleotide sequence having the sequence of SEQ ID NO: 67. The invention also provides a vector comprising a polynucleotide encoding the polypeptide comprising an amino acid having at least 95% sequence identity to the amino acid sequence of SEQ ID NO: (uvrD helicase) 68. The invention also provides a host cell comprising a vector comprising a nucleotide sequence encoding a polypeptide comprising an amino acid sequence having at least 95% sequence identity to the amino acid sequence of SEQ ID NO: (uvrD helicase) 68. In one embodiment, the polypeptide is a uvrD helicase from a thermophilic organism. In a different embodiment, the thermophilic organism is *Thermus thermophilus*.

The invention is also directed to an isolated polypeptide wherein said polypeptide comprises an amino acid sequence having at least 95% sequence identity to the amino acid sequence of SEQ ID NO: (DNA-G Primase) 72. In one embodiment, the polypeptide has the amino acid sequence of SEQ ID NO: 72. The invention also provides an isolated polynucleotide molecule comprising a nucleotide sequence encoding a polypeptide comprising an amino acid sequence having at least 95% sequence identity to the amino acid sequence of SEQ ID NO: (DNA-G Primase) 72. In one embodiment, the isolated polynucleotide molecule comprises a nucleotide sequence having the sequence of SEQ ID NO: 71. The invention also provides a vector

- 55 -

comprising a nucleotide sequence encoding a polypeptide comprising an amino acid sequence having at least 95% sequence identity to the amino acid sequence of SEQ ID NO: (DNA-G Primase) 72. The invention also provides a host cell comprising the vector. In one embodiment, the isolated polypeptide is a DNA G primase from a thermophilic organism. In another embodiment, the thermophilic organism is *Thermus thermophilus*.

The invention also provides an isolated polypeptide wherein said polypeptide comprises an amino acid sequence having at least 95% sequence identity to the amino acid sequence of SEQ ID NO: (priA helicase) 76. In one embodiment, the polypeptide has the amino acid sequence of SEQ ID NO: 76. The invention also provides an isolated polynucleotide molecule comprising a nucleotide sequence encoding a polypeptide comprising an amino acid sequence having at least 95% sequence identity to the amino acid sequence of SEQ ID NO: (priA helicase) 76. In one embodiment, the isolated polynucleotide molecule comprises a nucleotide sequence having the sequence of SEQ ID NO: 75. The invention further provides a vector comprising a nucleotide sequence encoding a polypeptide comprising an amino acid sequence having at least 95% sequence identity to the amino acid sequence of SEQ ID NO: (priA helicase) 76. The invention provides a host cell comprising the vector. In one embodiment, the isolated polypeptide is a priA helicase from a thermophilic organism. In another embodiment, the thermophilic organism is *Thermus thermophilus*.

The invention provides an isolated polypeptide wherein said polypeptide comprises an amino acid sequence having at least 95% sequence identity to the amino acid sequence of SEQ ID NO: (delta subunit) 10. In one embodiment, the polypeptide has the amino acid sequence of SEQ ID NO: 10. The invention also provides an isolated polynucleotide molecule comprising a nucleotide sequence encoding a polypeptide having at least 95% sequence identity to the amino acid sequence of SEQ ID NO: (delta subunit) 10. In one embodiment, the isolated polynucleotide molecule has the sequence of SEQ ID NO: 9. The invention provides a vector comprising a nucleotide sequence

- 56 -

encoding a polypeptide comprising an amino acid sequence having at least 95% sequence identity to the amino acid sequence of SEQ ID NO: (delta subunit) 10. The invention provides a host cell comprising said vector. In one embodiment, the isolated polypeptide is a delta subunit from a thermophilic organism. In one embodiment, the thermophilic organism is *Thermus thermophilus*. The invention further provides an isolated antibody molecule, wherein said antibody specifically binds to at least one antigenic determinant on a polypeptide which comprises an amino acid sequence having at least 95% sequence identity to the amino acid sequence of SEQ ID NO: (delta subunit) 10.

The invention provides an isolated polypeptide wherein said polypeptide comprises an amino acid sequence having at least 95% sequence identity to the amino acid sequence of SEQ ID NO: (delta prime subunit) 17. In one embodiment, the polypeptide has the amino acid sequence of SEQ ID NO: 17. The invention is further directed to an isolated polynucleotide molecule comprising a nucleotide sequence encoding a polypeptide comprising an amino acid sequence having at least 95% sequence identity to the amino acid sequence of SEQ ID NO: (delta prime subunit) 17. In one embodiment, the isolated polynucleotide molecule has the sequence of SEQ ID NO: 16. The invention also provides a vector comprising a nucleotide sequence encoding a polypeptide comprising an amino acid sequence having at least 95% sequence identity to the amino acid sequence of SEQ ID NO: (delta prime subunit) 17. The invention further provides a host cell comprising the vector. In one embodiment, the isolated polypeptide is a  $\delta'$  subunit from a thermophilic organism. In another embodiment, the thermophilic organism is *Thermus thermophilus*. The invention further provides an isolated antibody molecule, where in said antibody specifically binds to at least one antigenic determinant on the polypeptide which comprises an amino acid sequence having at least 95% sequence identity to the amino acid sequence of SEQ ID NO: (delta prime subunit) 17.

- 57 -

The invention is directed to an isolated polypeptide wherein said polypeptide comprises an amino acid sequence having at least 95% sequence identity to the amino acid sequence of SEQ ID NO: (beta subunit) 23. In one embodiment, the polypeptide has the amino acid sequence of SEQ ID NO: 23.

5 The invention is also directed to an isolated polynucleotide molecule comprising a nucleotide sequence encoding a polypeptide comprising an amino acid sequence having at least 95% sequence identity to the amino acid sequence of SEQ ID NO: (beta subunit) 23. In one embodiment, the isolated polynucleotide molecule has the sequence of SEQ ID NO: 22. The invention  
10 further provides a vector comprising a nucleotide sequence encoding a polypeptide comprising an amino acid sequence having at least 95% sequence identity to the amino acid sequence of SEQ ID NO: (beta subunit) 23. The invention also provides a host cell comprising the vector. In one embodiment, the isolated polypeptide of is a  $\delta'$  subunit from a thermophilic organism. In  
15 another embodiment, the thermophilic organism is *Thermus thermophilus*. The invention further provides an isolated antibody molecule, wherein said antibody specifically binds to at least one antigenic determinant on a polypeptide comprising an amino acid sequence having at least 95% sequence identity to the amino acid sequence of SEQ ID NO: (beta subunit) 23.

20 The invention is directed to an isolated polypeptide wherein said polypeptide comprises an amino acid sequence having at least 95% sequence identity to the amino acid sequence of SEQ ID NO: (ssb protein) 32. In one embodiment, the polypeptide has the amino acid sequence of SEQ ID NO: 32. The invention is also directed to an isolated polynucleotide molecule  
25 comprising a nucleotide sequence encoding a polypeptide comprising an amino acid sequence having at least 95% sequence identity to the amino acid sequence of SEQ ID NO: (ssb protein) 32. In one embodiment, the isolated polynucleotide molecule has the sequence of SEQ ID NO: 31. The invention further provides a vector comprising a nucleotide sequence encoding a  
30 polypeptide comprising an amino acid sequence having at least 95% sequence identity to the amino acid sequence of SEQ ID NO: (ssb protein) 32. The

- 58 -

invention provides a host cell comprising the vector. In one embodiment, the isolated polypeptide is an SSB protein from a thermophilic organism. In another embodiment, the thermophilic organism is *Thermus thermophilus*. The invention further provides an isolated antibody molecule, wherein said antibody specifically binds to at least one antigenic determinant on the polypeptide which comprises an amino acid sequence having at least 95% sequence identity to the amino acid sequence of SEQ ID NO: (ssb protein).

The invention is directed to an isolated polypeptide wherein said polypeptide comprises an amino acid sequence having at least 95% sequence identity to the amino acid sequence of SEQ ID NO: (epsilon-1, dnaQ-1) 37. In one embodiment, the polypeptide has the amino acid sequence of SEQ ID NO: 37. The invention is further directed to an isolated polynucleotide molecule comprising a nucleotide sequence encoding a polypeptide comprising an amino acid sequence having at least 95% sequence identity to the amino acid sequence of SEQ ID NO: (epsilon, dnaQ-1) 37. In one embodiment, the isolated polynucleotide molecule has the sequence of SEQ ID NO: 36. The invention also provides a vector comprising a nucleotide sequence encoding a polypeptide comprising an amino acid sequence having at least 95% sequence identity to the amino acid sequence of SEQ ID NO: (epsilon, dnaQ-1) 37. The invention further provides a host cell comprising the vector. In one embodiment, the isolated polypeptide is an epsilon-1 subunit from a thermophilic organism. In another embodiment, the thermophilic organism is *Thermus thermophilus*. The invention further provides an isolated antibody molecule, where in said antibody specifically binds to at least one antigenic determinant on a polypeptide which comprises an amino acid sequence having at least 95% sequence identity to the amino acid sequence of SEQ ID NO: (epsilon, dnaQ-1) 37.

The invention is directed to an isolated polypeptide wherein said polypeptide comprises an amino acid sequence having at least 95% sequence identity to the amino acid sequence of SEQ ID NO: (dnaQ-2) 82. In one embodiment, the polypeptide has the amino acid sequence of SEQ ID NO: 82.

- 59 -

The invention is further directed to an isolated polynucleotide molecule comprising a nucleotide sequence encoding a polypeptide comprising an amino acid sequence having at least 95% sequence identity to the amino acid sequence of SEQ ID NO: (dnaQ-2) 82. In one embodiment, the isolated polynucleotide molecule has the sequence of SEQ ID NO: 81. The invention is further directed to a vector comprising a nucleotide sequence encoding a polypeptide comprising an amino acid sequence having at least 95% sequence identity to the amino acid sequence of SEQ ID NO: (dnaQ-2) 82. The invention is also directed to a host cell comprising the vector. In one embodiment, the isolated polypeptide is an epsilon-2 subunit from a thermophilic organism. In another embodiment, the thermophilic organism is *Thermus thermophilus*. The invention further provides an isolated antibody molecule, where in said antibody specifically binds to at least one antigenic determinant on a polypeptide which comprises an amino acid sequence having at least 95% sequence identity to the amino acid sequence of SEQ ID NO: (epsilon-2, dnaQ-2) 82.

The invention is directed to a method of producing a polypeptide encoded by a nucleotide sequence, wherein said polypeptide comprises an amino acid sequence having at least 95% sequence identity to the amino acid sequence of one of SEQ ID NOS: 68, 72, 76, 10, 17, 23, 32, 37, and 82, comprising culturing a host cell comprising said nucleotide sequence under conditions such that said polypeptide is expressed, and recovering said polypeptide.

The invention is also directed to a method of synthesizing DNA which comprises utilizing one or more polypeptides, said one or more polypeptides comprising an amino acid sequence having at least 95% sequence identity to an amino acid sequence selected from the group consisting of SEQ ID NOS: 68, 72, 76, 10, 17, 23, 32, 37 and 82. In one embodiment, the method further comprises providing in any order: a reaction mixture comprising components comprising template, and nucleotides, and incubating said reaction mixture for a length of time and at a temperature sufficient to obtain DNA synthesis. In



- 60 -

another embodiment of the method, the method further comprises an N-terminal linked peptide or a C-terminal linked peptide.

It is contemplated that purified DnaQ-1 protein (epsilon subunit 1) and DnaQ-2 (epsilon subunit 2) find use in PCR and other applications in which high fidelity DNA synthesis is required or desirable. Although an understanding of the mechanism is not necessary in order to use the present invention, DnaQ-1 protein or DnaQ-2 protein bind to the  $\alpha$  subunit of DNA polymerase III, and works with it to efficiently remove errors made by the DNA polymerase III.

It is also contemplated that DnaQ-1 or DnaQ-2 will find use in place of an adjunct proofreading polymerase in PCR and other amplification applications. For example, when combined in an amplification reaction with a DNA polymerase that lacks a proofreading exonuclease, the DnaQ-1 or DnaQ-2 will facilitate elongation of PCR product as it is capable of removing mismatches within the PCR product. Thus, it is contemplated that the present invention (DnaQ-1 or DnaQ-2) will find use in such applications as long-range PCR (*e.g.*, PCR involving 5-50 kb targets).

It is contemplated that the DnaN protein will find use in purification of the  $\beta$  subunit (*i.e.*, the critical subunit that permits pol III to catalyze a processive (*i.e.*, long-distance without dissociating) amplification reaction. DnaN is useful with pol III alone (*e.g.*,  $\alpha$  or  $\alpha$  plus  $\epsilon$ ) on linear templates in the absence of additional subunits, or it can be used with the DnaX complex, as well as with additional proteins (*e.g.*, single-stranded binding proteins, helicases, and/or other accessory factors), to permit very long PCR reactions.

It is contemplated that the  $\alpha$  subunit,  $\beta$  subunit,  $\delta$  subunit,  $\delta'$  subunit,  $\epsilon$ -1 subunit,  $\epsilon$ -2 subunit,  $\gamma$  subunit,  $\tau$  subunit, ssb protein, uvrD protein, dnaG protein, and priA protein will find use separately or together in PCR and other applications in which high fidelity DNA synthesis is required or desirable, such as, for example, very long PCR reactions (5-50 kb targets). It is further contemplated that the foregoing N-terminal or C-terminal linked subunits and proteins will find use separately or together in PCR and other applications in

- 61 -

which high fidelity DNA synthesis is required or desirable, such as for example, very long PCR reactions (5-50kb).

Existing PCR technology is limited by relatively non-processive repair-like DNA polymerases. The present invention provides a thermophilic replicase capable of rapid replication and highly processive properties at elevated temperatures. It is contemplated that the compositions of the present invention will find use in many molecular biology applications, including megabase PCR by removing the current length restrictions, long range DNA sequencing and sequencing through DNA with high secondary structure, as well as enabling new technological advances in molecular biology.

All patents and publications referred to herein are expressly incorporated by reference in their entirety.

## EXAMPLES

The following examples serve to illustrate certain preferred embodiments and aspects of the present invention and are not to be construed as limiting the scope thereof.

In the experimental disclosure which follows, the following abbreviations apply: g (gram); L (liter);  $\mu$ g (microgram); ml (milliliter); bp (base pair); °C (degrees Centigrade); kb or Kb (kilobases); kDa or kd (kilodaltons); EDTA (ethylenediaminetetraacetic acid); DTT (dithiothreitol); LB (Luria Broth); -mer (oligomer); DMV (DMV International, Frazier, NY); PAGE (polyacrylamide gel electrophoresis); SDS (sodium dodecyl sulfate); SDS-PAGE (sodium dodecyl sulfate polyacrylamide gel electrophoresis); SSPE (2x SSPE contains 0.36 mM NaCl, 20 mM  $\text{NaH}_2\text{PO}_4$ , pH 7.4, and 20 mM EDTA, pH 7.4; the concentration of SSPE used may vary), SOP media (20 g/l tryptone (Difco), 10 g/l yeast extract (Difco), 5 g/l NaCl, 2.5 g/l potassium phosphate, dibasic (Fisher), 1 g/l  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$  (Fisher), pH 7.2); TE buffer (10 mM Tris, 1 mM EDTA); 50 x TAE (242 g Tris base, 57.1 ml

- 62 -

glacial acetic acid, 100 ml 0.5 M EDTA pH 8.0); Blotto (10% skim milk dissolved in dH<sub>2</sub>O and 0.2% sodium azide); Gel Loading Dye (0.25% Bromophenol blue, 0.25% xylene cyanol, 25% Ficoll (Type 400) in dH<sub>2</sub>O); Pre-hybridization mix (50% Formamide, 5X SSPE, 1% SDS, 0.5% CARNATION<sup>TM</sup> non-fat dried milk, 10% skim milk, 0.2% Na Azide); FBS (fetal bovine serum); ABS, Inc. (ABS, Inc., Wilmington, DE); GeneCodes (GeneCodes, Ann Arbor, MI); Boehringer Mannheim (Boehringer Mannheim, Indianapolis, IN); Champion Industries (Champion Industries, Clifton, NJ); Organon (Organon Teknika Corp., Durham NC); Difco (Difco, Detroit, MI); Enzyco (Enzyco Inc., Denver, Co); Fisher Scientific (Fisher Scientific, Fair Lawn, NJ); FMC (FMC, Rockland, Maine); Gibco BRL (Gibco BRL Gaithersburg, MD); Hyclone (Hyclone, Logan UT); Intermountain or ISC (ISC BioExpress, Bountiful, Utah); Invitrogen (Invitrogen, Carlsbad, CA); Millipore (Millipore, Marlborough, MA); MJ Research (MJ Research, Watertown, MA); Molecular Probes (Molecular Probes, Eugene, OR); National Diagnostics (National Diagnostics, Manville, NJ); Pharmacia Biotech (Pharmacia Biotech., Piscataway, NJ); Promega (Promega Corp., Madison, WI); Qiagen (Qiagen, Chatsworth, CA); Sigma PE/ABI (Perkin Elmer Applied Biosystems Division, Foster City, CA); (Sigma, St. Louis, MO); Stratagene (Stratagene, LaJolla CA); Tecan (Tecan, Research Triangle Park, NC); Whatman (Whatman, Maidstone, England); Lofstrand Labs (Lofstrand Labs, Ltd., Gaithersburg, Maryland) and LSPI (LSPI Filtration Products, Life Science Products, Denver, CO); Irvine (Irvine Scientific, Irvine CA); and Jackson Labs (Jackson Labs, Bar Harbor, Maine).

In Examples in which a molecular weight based on SDS-PAGE gels is reported for a protein, the molecular weight values reported are approximate values.

- 63 -

## EXAMPLE 1

## Construction of Starting Vectors

## 5 Construction of pA1-CB-Cla-2

Plasmid pA1-CB-Cla-1 was described in U.S. Patent Application 09/151,888, incorporated herein by reference. For the pA1-CB-Cla-1 plasmid to be useful for expression of several of the *T. thermophilus* genes, modifications were needed. To remove a *Kpn*I restriction site downstream of the C-terminal biotin tag, pA1-CB-Cla-1 plasmid DNA was prepared. All plasmid DNA preparations listed here and below were purified using Promega's Wizard® and Wizard® Plus DNA Purification Systems according to instruction from manufacturer. The pA1-CB-Cla-1 DNA plasmids were digested with *Kpn*I. The resulting 3' and 5' overhanging ends were removed by filling in with Klenow fragment and resealed with T4 DNA ligase in the presence of 1 mM ATP. Plasmids were transformed into DH5α, and plasmid-containing colonies were selected for ampicillin-resistance. Growth of starting vector are in 2xYT culture media (16 g/L bacto-tryptone, 10 g/L bacto-yeast extract, 5 g/L NaCl (pH 7.0) here and in following sections. Destruction of the *Kpn*I site in these plasmids was confirmed by DNA sequencing (ATG seq.# 630-631; primers P64-A215 and P38-S5576). One of the colonies that contained isolates that could not be cleaved by *Kpn*I was selected, grown, and used for preparation of the intermediate plasmid pA1-CB-Cla1(*Kpn*<sup>-</sup>) (ATG glycerol stock #424). Subunits of *T. thermophilus* DNA polymerase III holoenzyme were expressed in *E. coli* host cells. Nucleic acid (plasmids) may be introduced into bacterial host cells by a number of means including transformation of bacterial cells made competent for transformation by treatment with calcium chloride or by electroporation. A review of the use of transformation techniques is provided in Sambrook et al., Molecular Cloning: A Laboratory Manual, 2nd ed., Cold Spring Harbor Laboratory Press, New

- 64 -

York (1989) pp.1.74-1.84. The strategy used to introduce plasmids into DH5 $\alpha$  bacteria here is also used in all following similar transformation reactions.

The plasmid pA1-CB-Cla1(Kpn<sup>-</sup>) was digested with the restriction endonucleases *Cla*I and *Spe*I to remove the polylinker containing the restrictions sites : *Eag*I, *Bam*HI, *Xho*I, *Xba*II and *Dra*III. Two oligonucleotides (ATG linker/adaptor #P67-S1 and P67-A1) were annealed to form the adaptor/linker (shown below) (SEQ ID NO:1).

5'-CGATA AAAAAAAAAAGG CCGGCCGCTA GCGGTACCA-3'  
3'-TAT TTTTTTTTCC GGCCGGCGAT CGCCATGGTG ATC-5'

This adaptor/linker contained *Cla*I and *Spe*I sticky ends to allow insertion into these restriction sites present on the plasmid pA1-CB-Cla1 (Kpn<sup>-</sup>). The introduction of this adaptor/linker into *Cla*I/*Spe*I digested pA1-CB-Cla1(Kpn<sup>-</sup>) formed a new polylinker containing the restriction sites *Cla*I-spacer-*Fse*I-*Nhe*I *Kpn*I-*Spe*I and resulted in a new plasmid pA1-CB-Cla-2. This plasmid was transformed into DH5 $\alpha$  and plasmid containing colonies were selected by ampicillin-resistance. Plasmids were isolated from one positive clone and the sequence of the inserted DNA was confirmed by DNA sequencing (ATG seq.# 649, primer P38-S5576). The isolate containing the confirmed pA1-CB-Cla-2 plasmid was grown and stored as a stock culture (ATG glycerol stock #440).

#### Construction of pA1-CB-Nco-1

To construct pA1-CB-Nco-1 pDRK-C was first modified (See, Kim, D.R. and McHenry,C.S. (1996) *J Biol Chem* **271**, 20690-20698). Plasmid pDRKC DNA was prepared and digested with *Kpn*I. The resulting recessed and overhanging 3' ends were blunted with Klenow fragment and the plasmid was resealed. Plasmids were transformed into DH5 $\alpha$  and plasmid-containing colonies were selected by ampicillin-resistance. The plasmids were prepared and screened for loss of the *Kpn*I site. One positive clone containing a plasmid

- 65 -

that could not be cleaved by *KpnI* was selected and the DNA sequence was confirmed by DNA sequencing (ATG SEQ # 627 and 632; primers P38-S5576 and P64-A215). This plasmid was named pDRK-C (*Kpn*-) and the isolate was stored as a glycerol stock culture (ATG glycerol stock #414).

5           The plasmid pDRK-C (*Kpn*-) was digested with restriction endonucleases *XbaI* and *SpeI* to remove the polylinker containing the restriction sites *NcoI*, *EagI*, and *DraIII*. Two oligonucleotides (ATG linker/adaptor #P63-S1 and P63-A1) were annealed to form the adaptor/linker (shown below) (SEQ ID NO:2).

10  
5' -CTAGAGGAGGTTAATTAACCATGGAAAAAAAAAGGTACCAAAAAAAAAAGGCCGGCCA-3'  
3' -TCCTCCAATTAATTGGTACCTTTTTTTTCCATGGTTTTTTTTTCCGGCCGGTGATC-5'

15           This adaptor/linker contained *XbaI* and *SpeI* sticky ends to allow insertion into the corresponding restriction sites present on the pDRK-C (*Kpn*-) plasmid. The plasmid containing the inserted region was resealed and transformed into DH5 $\alpha$ . The introduction of this adaptor/linker into pDRK-C (*Kpn*-) formed a new polylinker containing the restriction sites *XbaI*-*PacI*-*NcoI*-spacer-*KpnI*-spacer-*FseI*-*SpeI*. The resulting ampicillin-resistant clones  
20           were screened for introduction of a *KpnI* restriction site. The plasmid from one positive clone was sequenced and was found to have the correct sequence in the region of the inserted linker/adaptor (ATG SEQ # 646 and 647; primers p38-S5576 and P65-A106). This plasmid was named pA1-CB-*Nco*-1. This isolate was grown and stored as a stock culture (ATG glycerol stock #438).

25           Construction of pA1-CB-*Nsi*1

30           To prepare the pA1-CB-*Nsi*1 plasmid, pA1-CB-*Nco*-1 was digested with restriction endonucleases *PacI* and *KpnI* to remove the polylinker containing the restriction sites *PacI*-*NcoI*-spacer-*KpnI*. Two oligonucleotides (ATG linker/adaptor #P68-S1 and P68-A1) were annealed to form the adaptor/linker (shown below) (SEQ ID NO:3).

- 66 -

5' -TTAAATGCATAAAAAAAAAAGGTAC-3'

3' -TAATTACGTATTTTTTTTTC-5'

5 This adaptor/linker contained *PacI* and *KpnI* sticky ends to allow  
insertion into the corresponding *PacI/KpnI* digested pA1-CB-Nco-1 plasmid.  
The plasmid was resealed and transformed into DH5 $\alpha$ . Introduction of this  
adaptor/linker into pA1-CB-Nco-1 formed a new polylinker containing the  
restriction sites *XbaI-PacI-NsiI*-spacer-*KpnI*-spacer-*FseI-SpeI*. The only  
10 change was replacement of the *NcoI* restriction site with an *NsiI* restriction  
site. The resulting clones were selected for ampicillin-resistance and isolated  
plasmids were screened for introduction of an *NsiI* restriction site. The  
plasmid from one positive isolate was sequenced and was found to have the  
correct sequence in the region of the inserted linker/adaptor (ATG SEQ # 663,  
15 primer P65-A106). This plasmid was named pA1-CB-Nsi-1 and the isolate  
was grown and stored as a stock culture (ATG glycerol stock #445).

#### Construction of pA1-CB-NdeI

20 To construct plasmid pA1-CB-NdeI, pA1-CB-NcoI was digested with  
*NdeI*. The overhanging ends were blunted with Klenow fragment to destroy  
the *NdeI* restriction site outside of the polylinker region. The linear plasmid  
was resealed forming pA1-CB-NcoI(NdeI-). This plasmid was transformed  
into DH5 $\alpha$  and plasmids were isolated from one resulting ampicillin-resistant  
25 colony. The plasmids were screened for loss of a *NdeI* site. The region filled in  
by Klenow fragment was sequenced to confirm the loss of the *NdeI* site (ATG  
SEQ 661, primer P65-S2529). pA1-CB-NcoI(NdeI-) was digested with *PacI*  
and *SpeI* restriction enzymes. This removed the polylinker containing *PacI*-  
*NcoI*-spacer-*KpnI*-spacer-*FseI-SpeI* restriction sites. An annealed DNA duplex  
30 or adaptor/linker (shown below) (SEQ ID NO:4) containing *PacI* and *SpeI*  
sticky ends (ATG linker/adaptor P65-S1 and P65-A1) was inserted into the  
digested pA1-CB-NcoI(NdeI-) plasmid.

- 67 -

5' -TAACATATGAAAAAAAAAACCAGGTTGCTAGCGGTACCA-3'  
 3' -TAATTGTATACTTTTTTTTTTTGGTCCAACGATCGCCATGGTGATC-5'

5 The introduction of this adaptor/linker into pA1-CB-NcoI(NdeI) formed a new polylinker containing the restriction sites *PacI*-*NdeI*-spacer-*NheI*-*KpnI*-*FseI*-*SpeI*. This plasmid was transformed into DH5 $\alpha$  and the plasmids were isolated from one resulting ampicillin-resistant colony. These plasmids were screened for the introduction of a *NdeI* site. The region  
 10 containing the inserted sequence was subjected to DNA sequencing to confirm insertion of the correct sequence (ATG SEQ #718, primer P38-S5576). This plasmid was named pA1-CB-NdeI and the positive isolate was grown and stored as a stock culture (ATG glycerol stock #464)

#### 15 Construction of pA1-NB-Avr-2

To construct pA1-NB-Avr-2, DRK-N(M), a plasmid designed for expression of proteins with an amino-terminal tag was used as the starting plasmid. The amino-terminal tag is composed of a 30 amino acid peptide that  
 20 is biotinylated *in vivo*, a hexahistidine site, and thrombin cleavage site (*See*, Kim and McHenry, J. Biol. Chem., 271:20690-20698 [1996]). Also, there is a pBR322 origin of replication, a gene expressing the *laqI*<sup>Q</sup> repressor protein, and a semisynthetic *E. coli* promoter (pA1) that is repressed by the *lacl*<sup>Q</sup> repressor.

25 The following two oligonucleotides were separately synthesized, annealed to form a duplex with sticky ends (*AvrII* and *SalI*), and inserted into the *AvrII*/*SalI* digested pDRK-N(M). The synthetic linker/adaptor consisted of two annealed oligonucleotides (ATG linker/adaptor P64-S1 and P64-A1) (shown below) (SEQ ID NO:5).

30 5' -CTAGGAAAAAAAAAGGTACCAAAAAAAAAAGGCCGCCACTAGTG-3'  
 3' -CTTTTTTTTTTCCATGGTTTTTTTTTCCGGCCGGTGATCACAGCT-5'



- 68 -

The insertion of these annealed DNA fragments into pDRK-N(M) converted the polylinker following the fusion peptide from *AvrII*-*DraIII*-*SalI* to *AvrII*--spacer--*KpnI*--spacer--*FseI*--*SpeI*--*SalI*. These plasmids were transformed into DH5 $\alpha$  and the resulting ampicillin-resistant colonies were screened for plasmids that contained a *SpeI* site carried by the linker/adaptor. One positive clone was selected and the sequence of the inserted region was confirmed by DNA sequencing across the linker/adaptor region (ATG SEQ #648, primer P64-A215). This plasmid was named pA1-NB-Avr-2 and the isolate was grown and stored as a glycerol stock culture (ATG glycerol stock #439).

#### Construction of pA1-NB-Kpn1

The pA1-NB-Avr-2 plasmid was modified to construct pA1-NB-Kpn1 by replacing the polylinker containing the *AvrII*--spacer--*KpnI*--spacer--*FseI*--*SpeI*--*SalI* with a polylinker containing the restriction sites *PstI*-*KpnI*-Spacer-*NsiI*-*SacI*-*NheI*-*HindIII*-spacer-*SpeI*. This was accomplished by digestion of pA1-NB-Avr-2 with *PstI* and *SpeI* restriction enzymes and insertion of the annealed DNA duplex shown below (ATG adaptor/linker # P64--S1 and P64--A1). The ends of the annealed duplex DNA formed sticky ends corresponding to *PstI*/*SpeI* restriction sites (shown below) (SEQ ID NO:6).

5' -GGTACCAAAAATGCATGAGCTCGCTAGCAAGCTTAAAAAAAAA-3'

3' -ACGTCCATGGTTTTTACGTACTCGAGCGATCGTTTCAATTTTTTTTTTGATC-5'

The first spacer allows *PstI*/*NsiI* double digests and the last spacer allows *HindIII*/*SpeI* double digests. The plasmids were transformed into DH5 $\alpha$  bacteria and ampicillin-resistant colonies were screened for plasmids that contained *HindIII* restriction site carried by the linker/adaptor. The DNA sequence of the linker/adaptor region was confirmed by DNA sequencing

- 69 -

(ATG SEQ #662, primer P64-A215). This plasmid was named pA1-NB-Kpn-1 and the isolate was grown and stored as a glycerol stock culture (ATG glycerol stock #446).

## 5 Construction of pA1-NB-AgeI

The pA1-NB-Avr-2 plasmid was modified to construct pA1-NB-AgeI. This was done by replacing the polylinker in pA1-NB-Avr-2 which contained the restriction sites *PstI*-*AvrII*-*KpnI*-*FseI*-*SpeI* with a polylinker containing the  
 10 restriction sites *PstI*-spacer-*AgeI*-*BamHI*-*SacII*-spacer-*NcoI*-*SpeI*. First, a *BamHI* site upstream of the polylinker was destroyed. This was accomplished by digesting pA1-NB-Avr-2 with *BamHI* and filling in the sticky ends created by the digestion with Klenow fragment. The blunted ends of the DNA were  
 15 resealed. The plasmid was transformed into DH5 $\alpha$  and positive isolates were selected by ampicillin-resistance. Plasmids were isolated from one positive isolate and were screened for by the loss of the *BamHI* restriction site. The loss of the *BamHI* restriction site was confirmed by DNA sequencing (ATG SEQ #1171, primer P64-A215). This plasmid was named pA1-NB-Avr2(*BamHI*) and the positive isolate was stored as a stock culture (ATG  
 20 glycerol stock #688).

pA1-NB-Avr2(*BamHI*) was digested with *PstI*/*SpeI* restriction enzymes. This removed the polylinker containing the restriction sites *PstI*-*ArvI*-*KpnI*-*FseI*-*SpeI*. An annealed duplex (ATG adaptor/linker #P116-S1 and P116-A1) (shown below) was inserted into digested pA1-NB-Avr2(*BamHI*)  
 25 (SEQ ID NO:7).

5' -GAAAAAAAAAACCGGTGGATCCGCGGAAAAAAAAACCATGGA-3'

3' -ACGTCTTTTTTTTTTGGCCACCTAGGCGCCTTTTTTTGGTACCTGATC-5'

30 The ends of the annealed duplex DNA forms sticky ends cooresponding to *PstI* and *SpeI* restriction sites. This plasmid was transformed

- 70 -

into DH5 $\alpha$  and plasmids isolated from the growth of one clone were screened for by the ability to be digested with *Age*I, *Bam*HI, *Sac*II and *Nco*I restriction enzymes. The sequence of the inserted region in this plasmid was confirmed by DNA sequencing (ATG SEQ #1176, primer #P64-A215). This plasmid was named pA1-NB-AgeI and the positive isolate was stored as a stock culture (ATG glycerol stock #698).

#### Construction of pTAC-CCA-ClaI

In an attempt to express native proteins from *T. thermophilus* in *E. coli* that have not expressed well, a vector system was constructed that can be used to express proteins as translationally coupled proteins. Plasmid (pTACCCA (pTC9) contains a gene encoding *E. coli* ATP(CTP):tRNA nucleotidyl transferase (referred to as CCA adding enzyme) under control of a *tac* promoter. This gene is expressed at very high levels. All of this gene was removed except the 5' 12 codons so that the *T. thermophilus* *dnaE* gene could be coupled to this remaining 5' end as a translationally coupled protein (pTAC-CCA-TE) (discussed below). Beginning with the plasmid pTAC-CCA-TE, a plasmid was designed containing a polylinker that will allow insertion of other target proteins that can be expressed as translationally coupled proteins. First, pTAC-CCA-TE was digested with *Nsi*I and *Spe*I. The *Nsi*I restriction site is approximately 35 nucleotide downstream of the CCA adding enzyme start ATG and the *Spe*I is downstream of the *T. thermophilus* *dnaE* stop TAG. This removed the entire *T. thermophilus* *dnaE* (TE) gene and the region linking the CCA adding enzyme gene 5' end to the TE gene. Next, the annealed DNA duplex (below) (SEQ ID NO:8) (ATG adaptor/linker #P152-SL and P152-AL), containing *Nsi*I and *Spe*I sticky ends was inserted into the digested pTAC-CCA-TE plasmid.

5' -TTGAGGAGGTATCGataaAAAAACCGGTCCTAGGCTAGCTCGAGA-3'  
3' -ACGTAACCTCCTCCATAGCTATTTTTTTGGCCAGGATCCGATCGAGCTCTGATC-5'

- 71 -

This DNA duplex contains “AGGAGG” (*italics*), the ribosome binding site (RBS), downstream of the *NsiI* sticky end, followed by a *ClaI* restriction site (underlined) for insertion of the 5' end of target genes. The *ClaI* restriction site contains the “t” of the “taa” stop (lower case) for terminating translation of the CCA adding enzyme gene 5' end including the linker region. The added sequence provided by the adaptor/linker (including the ribosome binding site and *ClaI* restriction site) is such that codon maintenance is in frame with the CCA adding enzyme gene 5' end up to the “taa” stop codon. Here and in the remainder of the text, when a region of DNA is addressed as being “in frame” with another DNA region, this indicates that the codon maintenance for the two regions is such that continued protein expression (translation) is possible without encountering a “stop” codon and therefore terminating the synthesis of the protein. The second “a” of the stop will be used to form the first nucleotide of the “ATG” start codon of the target translationally coupled gene, which is out of frame with the CCA adding enzyme. The remainder of the adaptor/linker contains a polylinker containing the restriction sites *ClaI*-*taa*-spacer-*AgeI*-*AvrII*-*NheI*-*XhoI*-*SpeI* to accommodate internal restriction sites or sites downstream of stop codons for insertion of target genes. This plasmid was transformed into DH5 $\alpha$  and plasmid containing colonies were selected for by ampicillin-resistance. One positive colony was selected and the isolated plasmids were screened for by digesting with *NsiI*, *ClaI* and *SpeI* giving single cuts resulting in linear fragments (5.5 kb). The sequence of the inserted region in this plasmid was confirmed by DNA sequencing (ATG SEQ #1617, primer #P144-S23). This plasmid was named pTAC-CCA-*ClaI* and the positive isolate was grown and stored as a stock culture (ATG glycerol stock #980).

Target genes will be amplified using PCR in which the forward/sense primer contains ATCGATAatg..... . The underlined sequence will be complementary to the 5' end of the target gene, while the upper case is non-complementary and contains the *ClaI* site needed for insertion into pTAC-CCA-*ClaI*. Adjacent to the *ClaI* site is the 5' TA of the stop codon. The “*a*” (*italics*) corresponds to the final “a” of the stop “TAA” and also to the “a” of

- 72 -

the start "atg" which are overlapping. The reverse/antisense primer must include one of the restriction sites in the polylinker region to allow insertion of the 3' end of the target gene into pTAC-CCA-ClaI. The mechanism of translationally coupling is that the messenger RNA (mRNA) of a highly expressed protein (CCA adding enzyme) is partially translated and then the ribosome encounters the premature stop codon. The inserted RBS inhibits disengagement of the ribosome from the mRNA until the ribosome recognizes the new start codon and proceeds to translate the target protein. Our assumption is that the ribosome RNA helicase activity disrupts secondary structure in the GC-rich *T. thermophilus* sequences, permitting more efficient translational initiation.

## EXAMPLE 2

Verification of Expression of *T. thermophilus*  $\alpha$ -subunit Fused to an N-Terminal Peptide That Contains Hexahistidine and a Biotinylation Site by pA1-NB-TE/MGC1030

In U.S. Patent Application No. 09/151,888, the cloning of *Tth dnaE* gene into the pA1-NB-Avr-2 and transformation into MGC1030 was described. Insertion of the *dnaE* gene into this vector allows the  $\alpha$ -subunit to be expressed as an N-terminal tagged protein. The verification of expression was as described below.

PA1-NB-TE was transformed into MGC1030 *E. coli* bacteria (mcrA, mcrB, lamBDA(-), (RRND-RRNE)1, lexA3) (ATG glycerol stock #938) and AP1.L1 *E. coli* (ATG glycerol stock #939). The parent to the AP1.L1 bacterial strain was Novagen BLR bacterial strain [F-, ompT hsdSB(rB- mB-) gal dcm,(srl-recA)306::Tn10. A T1 phage-resistant version of this BLR strain was designated AP1.L1. Single colonies (3 colonies from each transformation) of transformed cells selected for by ampicillin-resistance were inoculated into 2 ml of 2xYT culture media containing 100  $\mu$ g/ml ampicillin and grown overnight at 37°C in a shaking incubator. In the morning, 0.5 ml of the turbid culture from the overnight growth was inoculated into 1.5 ml of

- 73 -

fresh 2xYT culture media. The cultures were grown for 1 hour at 37°C with shaking and expression was induced by addition of isopropyl- $\beta$ -D-thiogalactopyranoside (IPTG) to a final concentration of 1 mM. The cells were harvested by centrifugation 3 hours post-induction. The cell pellets were immediately resuspended in 1/10 culture volume of 2x Laemmli sample buffer (2x solution: 125 mM Tris-HCl (pH 6.8), 20% glycerol, 4% sodium dodecyl sulfate (SDS), 5%  $\beta$ -mercaptoethanol, and 0.005% bromophenol blue w/v), and sonicated to complete lysis of cells and to shear the DNA. The samples were heated for 10 minutes at 90-100°C, and centrifuged to remove insoluble debris. A small aliquot of each supernatant (3  $\mu$ l) containing total cellular protein was electrophoresed onto a 4-20% SDS-polyacrylamide mini-gel (Novex, EC60255; 1 mm thick, with 15 wells/gel) in 25 mM Tris base, 192 mM glycine, and 0.1% SDS. The mini-gels were stained with Coomassie Blue. A protein migrating just above the 120 kDa molecular weight standard of the Gibco 10 kDa protein ladder could be detected as a distinct protein band, but was not observed in the uninduced control. This protein band corresponds to the expected molecular weight of the *T. thermophilus*  $\alpha$ -subunit fused to the N-terminal fusion protein (141 kDa).

Next, the total protein in each lysate was transferred (blotted) from polyacrylamide gel to nitrocellulose. The total protein in each lysate was transferred (blotted) from polyacrylamide gel to nitrocellulose membrane using a Novex transfer apparatus at 30 V constant voltage in 12 mM Tris base, 96 mM glycine, 0.01% SDS (w/v), and 20% methanol (v/v) for 60 minutes at room temperature. The membrane was blocked in 0.2% Tween 20 (v/v)-TBS (TBST) (tris-buffered saline; 8 g/L NaCl, 0.2 g/L KCl, 3 g/L Tris-HCl (pH 7.4)) containing 5% non-fat dry milk (w/v) for 1 hour at room temperature. The blotted nitrocellulose was next rinsed TBST, and then incubated in 2  $\mu$ g/ml alkaline phosphatase-conjugated streptavidin (Pierce Chemical Co. #21324) in TBST for 1 hour at room temperature. Following extensive washing TBST, the blot was developed with BCIP/NBT (KPL #50-81-07; one component system). The endogenous *E. coli* biotin-carboxyl carrier protein

- 74 -

(biotin-CCP), ~20 kDa was detectable in both induced and non-induced samples. A very intense protein band corresponding to  $\alpha$  migrated above the 140 kDa molecular weight standards of the Gibco 10 kDa protein ladder. This protein was observed as a distinct band in the induced cultures, but was not  
5 observed in the uninduced control.

The procedures described here to verify protein expression, which includes lysing cells, obtaining total cellular protein and analysing the protein in SDS-polyacrylamide gel electrophoresis and biotin blot analysis will be used in all following procedures to verify expression of native and tagged  
10 proteins. All protein concentrations here and below are determined using the Coomassie Protein Assay Reagent from Pierce and bovine serum albumin (BSA) as a standard.

#### Large Scale Growth of pA1-NB-TE/MGC1030

Strain pA1-NB-TE/MGC1030 was grown in a 250 L fermentor to produce cells for purification of *T. thermophilus*  $\alpha$  as described in the section entitled "Large Scale Growth of Native *T. thermophilus* *dnaE* ( $\alpha$ -subunit) by pTAC-CCA-TE". Cell harvest was initiated 3 hours after induction, at OD<sub>600</sub>  
20 of 7.2, and the cells were chilled to 10 °C during harvest. The harvest volume was 175 L, and the final harvest weight was approximately 2.47 kg of cell paste. An equal amount (w/w) of 50 mM Tris (pH 7.5) and 10% sucrose solution was added to the cell paste. Quality control results showed 10 out of 10 positive colonies on ampicillin-containing medium in the inoculum and  
25 10/10 positive colonies at harvest. Cells were frozen by pouring the cells suspension into liquid nitrogen, and stored at -20°C, until processed.

#### Purification of *T. thermophilus* $\alpha$ Fused to an N-terminal Peptide Containing a Hexahistidine and a Biotinylation Site

Lysis was accomplished by creation of spheroplasts of the cells carrying the expressed *T. thermophilus*  $\alpha$ -subunits. First, from 600 g of a 1:1  
30

- 75 -

suspension of frozen cells (300 g cells) in Tris-sucrose which had been stored at  $-20^{\circ}\text{C}$ , Fr I was prepared (875 ml, 21.6 mg/ml). The preparation was as described in the section entitled "Determination of Optimal Ammonium Sulfate Precipitation Conditions of *T. thermophilus*  $\alpha$ -subunit Expressed as a Translationally Coupled Protein." To Fr I, ammonium sulfate (0.258 g to each initial ml Fraction I-45% saturation) was added over a 15 min interval. The mixture stirred for an additional 30 min at  $4^{\circ}\text{C}$  and the precipitate was collected by centrifugation ( $23,000 \times g$ , 45 min,  $0^{\circ}\text{C}$ ). The resulting pellets were quick frozen by immersion in liquid nitrogen and stored at  $-80^{\circ}\text{C}$ .

The pellets from Fr I were resuspended in 90 ml of  $\text{Ni}^{++}$ -NTA suspension buffer and homogenized using a Dounce homogenizer. The sample was clarified by centrifugation ( $16,000 \times g$ ) and the supernatant constituted Fr II (98 ml, 25 mg/ml). Fr II was added to 50 ml of a 50% slurry of Ni-NTA resin and rocked for 1.5 hours at  $4^{\circ}\text{C}$ . This slurry was then loaded onto a BioRad Econo-column ( $2.5 \times 5 \text{ cm}$ ). The column was washed with 250 ml of  $\text{Ni}^{++}$ -NTA wash buffer at a flow rate of 0.5 ml/min. *T. thermophilus*  $\alpha$  was eluted in 150 ml of  $\text{Ni}^{++}$ -NTA elution buffer containing a 10-200 mM imidazole gradient. The eluate was collected in  $80 \times 2 \text{ ml}$  fractions (FIG. 1). Fractions 30-50 were pooled (see FIG. 1) and constitute FrIII (63ml, 2 mg/ml).

#### Construction of Plasmids (pTAC-CCA-TE) that Overexpress *T. thermophilus* $\alpha$ -Subunit as a Translationally Coupled Protein

In the preceeding patent application (U.S. Application # 09/151888) the *T. thermophilus dnaE* gene (TE) expressing the  $\alpha$ -subunit was cloned into pA1-CB-NcoI resulting in the plasmid pA1-TE. This plasmid was designed to express the native form of the  $\alpha$ -subunit, but yields of the  $\alpha$ -subunit were at very low levels (as previously discussed). In an attempt to increase the level of expression of the native  $\alpha$ -subunit a vector was designed to express the  $\alpha$ -subunit as a translationally coupled protein. Translational coupling with an upstream highly expressed protein will be used to disrupt strong secondary structures present in the GC-rich *T. thermophilus dnaE* mRNA, permitting



- 76 -

more efficient translational initiation and higher levels of *T. thermophilus* expression. The starting plasmid was pTACCCA (pTC9) and contained the CCA adding enzyme under control of a pTAC promoter. This plasmid expresses the CCA adding enzyme at high levels. The strategy was to remove most of the CCA adding enzyme leaving only the 5'-12 codons by digesting pTACCCA plasmid with *Nsi*I and *Kpn*I. The *Nsi*I restriction site is approximately 12 codons downstream of the ATG start site of the CCA adding enzyme and the *Kpn*I restriction site is downstream of the stop codon.

The TE gene was inserted behind the CCA adding enzyme and translationally coupled in two steps. First, the 5' end of the TE gene was amplified using pA1-TE as a template by polymerase chain reaction (PCR). The forward primer (ATG primer #P69-S541) is shown below.

5'-GGATATGCATTGAGGAGGATCGATTAAatgggcccgc aaactccgc-3'

(SEQ ID NO:20)

The non-complementary portion of the primer is shown as upper case and the portion of the primer complementary to the 5' end of the gene is shown as lower case. The *Nsi*I site (ATGCAT) and the *Cla*I site (ATCGAT) are shown as underlined italic. The RBS (AGGAGG) is shown as underlined. Both the RBS and the *Cla*I restriction site maintain codons that are inframe with the structural gene for the CCA adding enzyme. The last two nucleotides of the non-complementary portion of the primer "TA" and the first nucleotides of the complementary portion of the primer "a" form a premature stop codon, in frame with the 5' end of the CCA adding enzyme. The "a" also is the first nucleotide of the "atg" start codon of the TE gene. This places the gene for the CCA adding enzyme and the TE gene out of frame with respect to each other. The sequence of the reverse primer (5'-CGGCTCGCCAGGCGCACCAGG-3') (SEQ ID NO:21) (ATG primer #P69-A971) is complementary to a region just down stream of a unique *Kpn* I site located approximately 316 bp downstream of the start "ATG" codon.

- 77 -

The PCR product resulting from the forward and reverse primers described in the preceding paragraph (430 base pairs in length) was cut with *NsiI* and *KpnI* yielding a 350 bp fragment and inserted into the *NsiI/KpnI* digested pTACCCA plasmid. By cutting the pTACCCA plasmid with these two enzymes the C-terminal (3') *ca.* 95% of the CCA adding enzyme gene along with approximately 600 bp of sequence downstream of the stop codon was removed. The resulting plasmid was transformed into DH5 $\alpha$  and positive isolates were selected by ampicillin-resistance. Plasmid isolated from one positive isolate was verified by digestion with *NsiI* and *KpnI* (yielding the expected 0.35 and 5.3 kb fragments). The sequence of the insert was confirmed by DNA sequencing (ATG SEQ #1512 and 1513, primers #P144-S23 and P144-A1965, respectively). This plasmid was named pTAC-CCA-TEmp and the isolate was stored as a glycerol stock culture (ATG glycerol stock #898).

To reconstruct the remainder of the *T. thermophilus dnaE* gene, the pA1-TE plasmid was digested using the restriction enzymes *KpnI* and *SalI*. The *SalI* restriction site is approximately 254 bp downstream of the end of the TE gene. It is also located downstream of a C-terminal biotin-hexahistidine fusion peptide. The resulting 3601 base pair *KpnI-SalI* fragment encompassing the C-terminal (3') 95% of the *T. thermophilus dnaE* gene, was inserted into the *KpnI/SalI* digested pTAC-CCA-TEmp plasmid. The plasmid was ligated, transformed into DH5 $\alpha$  and positive isolates were selected for ampicillin-resistance. Plasmid isolated from one positive isolate was verified by digestion with *KpnI* and *SalI* restriction enzymes (yielding the expected 3.6 and 5.6 kb fragments). The sequence of the insert was confirmed by DNA sequencing (ATG SEQ #1550 and 1551, primers #P144-S23 and P144-A1965, respectively). This plasmid was named pTAC-CCA-TE and the isolate pTAC-CCA-TE/ DH5 $\alpha$  was stored as a glycerol stock culture (ATG glycerol stock #933).

- 78 -

### Verification of Expression of Native *T. thermophilus* *dnaE* gene ( $\alpha$ -subunit) as a Translationally Coupled Protein by pTAC-CCA-TE

pTAC-CCA-TE plasmids were transformed into MGC1030 (ATG glycerol stock #938) and AP1.L1 *E. coli* (ATG glycerol stock #939). Three isolates from each transformation were grown and total protein isolated as described above. An aliquot (3  $\mu$ l) of each supernatant was subjected to electrophoresis in a 4-20% SDS-polyacrylamide mini-gel (Novex, EC60255; 1 mm thick, with 15 wells/gel) in 25 mM Tris base, 192 mM glycine, and 0.1% SDS. The resulting gels were stained with Coomassie Brilliant Blue. Distinct protein bands from both MGC1030 and AP1.L1 bacterial preparations, migrating slightly above the 120 kDa molecular weight standard, of the Gibco 10 kDa protein ladder, were observed as distinct bands in the induced cultures, but were not observed in the uninduced controls. These proteins were determined to be consistent with the expected molecular weight expected for native *T. thermophilus*  $\alpha$  (137.5 kDa). The detected proteins represented approximately 5% of the total *E. coli* protein, based on the intensity of Coomassie Blue staining of the protein bands on the gel.

### Optimization of *T. thermophilus* Protein Expression

In an attempt to optimize the yield of expressed recombinant *T. thermophilus* proteins, induction times were analyzed for each new protein. F-media (Bacto Yeast Extract, 14 g/L, Bacto Tryptone, 8 g/L, potassium phosphate-dibasic, 12 g/L, potassium phosphate-monobasic, 1.2 g/L, (pH 7.2), 1% glucose) is used as a growth medium. A small amount of F-media (10-20 ml) containing ampicillin is inoculated with the target bacteria and grown overnight at 37°C while shaking. This overnight growth is used to inoculate fresh F-media containing ampicillin pre-warmed to 37°C. The fresh media is inoculated at a 20:1 ratio using the culture grown overnight. This allows enough time for cell density to double 3-4 times before induction. The freshly inoculated culture is grown to an  $OD_{600} = 0.6-0.8$  (The optical density

- 79 -

(OD)<sub>600</sub> is a unit used to measure light scattered by cells in solution at 600 nanometers in calculating the density of cells in the solution) and expression induced by addition of IPTG to 1 mM. At the time of induction *d*-biotin is added to proteins containing hexahistidine and a biotinylation site to a final concentration of 10  $\mu$ M. The control culture received *d*-biotin only--they were not induced with IPTG.

Equal sample volumes (5ml) of culture are collected at the time of induction and every hour after induction up to 5 hours post induction for analysis to determine optimum growth times. The OD<sub>600</sub> of each sample is determined. The samples collected are centrifuged in a Fisher Centrifuge Model 228 (1380 x g) for 10 min. The supernatant is discarded and the cell pellets were retained for analysis. To maintain equal concentration of total protein in each sample, 50  $\mu$ l of Laemmli lysis buffer (125 mM Tris-HCl, (pH 6.8), 20% glycerol, 5% SDS) was added per OD<sub>600</sub> of each sample multiplied by the sample volumes (5 ml). The cell pellets are resuspended and heated to 90-100°C for 10 min. The samples are centrifuged at maximum rpm (16,000 x g) for 10 min using a table top microfuge, and the supernatant is retained. Small aliquots containing total cellular protein, of each supernatant (5  $\mu$ l) are loaded onto a 10% SDS-polyacrylamide gel electrophoresis gel (16 x 18 x 0.75 cm) in 25 mM Tris base, 192 mM glycine, and 0.1% SDS. The gels are electrophoresed for 2 h at 250 volts. The gels are stained with Coomassie Blue or (for proteins containing hexahistidine and a biotinylation site) transferred to nitrocellulose and analyzed by biotin blot analysis. Biotin blot analysis is used to refer to proteins that have been transferred from SDS-polyacrylamide gels to nitrocellulose membrane and proteins detected by virtue of biotin bound to an N- or C-terminal peptide that contains a biotinylation site. In normally growing cells a certain percentage of proteins containing a biotinylation site is bound by biotin. The detection of these proteins is by virtue of avidin binding to the biotin bound to the fusion peptide. Alkaline phosphatase-conjugated streptavidin (Pierce Chemical Co.

- 80 -

#21324) is used and can be detected using chemicals that allow the alkaline phosphatase and therefore the protein of interest to be visualized.

#### Optimization of Expression of *T. thermophilus* *dnaE* gene ( $\alpha$ -subunit) by pTAC-CCA-TE

In preliminary experiments, *T. thermophilus*  $\alpha$  appeared to be synthesized at higher levels in the AP1.L1 strain. Therefore, the optimum induction times for expression of *T. thermophilus* from pTAC-CCA-TE carried in AP1.L1 were analyzed. The yield of *T. thermophilus*  $\alpha$  was analyzed at 1, 2, 3, 4, and 5 h induction times as described above in section "Optimization of *T. thermophilus* Protein Expression. The optimum yield of *T. thermophilus*  $\alpha$  was attained by 3 h post induction; this induction time was used in subsequent experiments (FIG. 2).

#### Gap-Filling Assay for Determination of *T. thermophilus* $\alpha$ -subunit Activity

The catalytic subunit of a replicative complex has a very low processivity in the absence of other holoenzyme subunits on a primed-template. However, the catalytic subunit can fill the gaps of nuclease-activated (gapped) DNA very effectively by fast association and dissociation reactions in low salt conditions (shown below) (See, McHenry and Crow (1979), *J. Biol. Chem.*, 254, 1748-1753). To be able to assay for activity in different purification steps of *T. thermophilus*  $\alpha$ -subunit the gap-filling assay was used.

Assay mixtures (25 $\mu$ l) contained 32 mM Hepes (pH 7.5), 13% glycerol, 0.01% Nonidet P40, 0.13 mg/ml BSA, 10mM MgCl<sub>2</sub>, 0.2mg/ml activated calf-thymus DNA, 57uM each of dGTP, dATP, and dCTP, and 21 $\mu$ M [<sup>3</sup>H] TTP (approximately 100 cpm/pmol). The mixture was assembled on ice, and reactions were started by the addition of a dilution of samples of DNA polymerase and placing in a 60°C water bath for 5 minutes. The reactions were stopped by placing the tubes on ice and the DNA

- 81 -

precipitated by adding 2 drops of 0.2M sodium pyrophosphate (PPi) and 0.5 ml of 10% TCA. Trapping of precipitated DNA and removal of unincorporated nucleotide triphosphates was accomplished by filtering the mixture through GFC filters (Whatman) and washing the filters with 12 ml 0.2M sodium PPi/1M HCL and then 4 ml of ethanol. The filters were then allowed to dry and [<sup>3</sup>H]TTP incorporated was quantified by immersing the filters in 5 ml of liquid scintillation fluid (Ecoscint-O, National Diagnostics) and counting on a Beckman LS 3801 scintillation counter. One unit of enzyme activity is defined as one picomole of total nucleotides incorporated per min at 60°C. Positive controls, containing *E. coli* DNA pol III (assayed at 30°C), and negative controls, containing no polymerase, were included in each set of assays

#### Large Scale Growth of Native *T. thermophilus* $\alpha$ by pTAC-CCA-TE/AP1.L1

Strain pTAC-CCA-TE/AP1.L1 was grown in a 250 L fermentor to produce cells for purification of *T. thermophilus* *dnaE* product ( $\alpha$ ). F-medium (1.4% yeast extract, 0.8% tryptone, 1.2% K<sub>2</sub>HPO<sub>4</sub>, and 0.12% KH<sub>2</sub>PO<sub>4</sub>, pH to 7.2 with NaOH) was sterilized, glucose was added to 1% from a 40% sterile solution and ampicillin (100 mg/L) was added. A large-scale inoculum (28 L), was initiated from a 1 ml glycerol stock culture (*i.e.*, culture stored in 15% glycerol at -80°C) and grown overnight at 37°C with 40 L/min aeration. The inoculum was transferred (approximately 4.2 L) to the 250 L fermentor containing 180 L of F-medium with 1% glucose, and 100 mg/L ampicillin (starting OD<sub>600</sub> of 0.06). To calculate the amount of overnight culture to add to the fermentor, in this fermentation there was 180 L initial F-media, enough should be added to bring the media present in the fermentor to an OD<sub>600</sub> = 0.06. This allows enough time for the cell density to double 3-4 times before induction. The culture was incubated at 37°C, with 40 LPM aeration, and stirred at 20 rpm. Expression of *T. thermophilus*  $\alpha$  was induced by addition of IPTG to 1 mM when the culture reached an OD<sub>600</sub> of 0.79 (expression of

- 82 -

foreign proteins in *E. coli* is induced when the cell density reaches approximately an  $OD_{600}$  = 0.6-0.8). Additional ampicillin (100 mg/L) was added at same time as induction. The temperature was maintained at approximately 37°C throughout the growth. The pH was maintained at 7.2 throughout the growth by addition of  $NH_4OH$ . Cell harvest was initiated 3 hours after induction at  $OD_{600}$  = 4.88, and the cells were chilled to 10°C during harvest. The harvest volume was 180 L, and the final harvest weight was approximately 1.9 kg of cell paste. An equal amount (w/w) of 50 mM Tris (pH 7.5) and 10% sucrose solution was added to the cell paste. Cells were frozen by pouring the cells suspension into liquid nitrogen, and stored at -20°C, until processed. Quality control results showed 10 out of 10 positive colonies on ampicillin-containing medium in the inoculum and 10 out of 10 positive colonies on ampicillin-containing medium at harvest. Positive colonies are colonies grown from samples streaked on LB plates that also grow when the colony is transferred to LB plates containing a selective antibiotic. Luria-Bertani (LB) growth medium (bacto-tryptone, 10 g/L, bacto-yeast extract, 5 g/L, NaCl, 10 g/L) is used in selection of positive colonies here and in following sections.

#### Determination of Optimal Ammonium Sulfate Precipitation Conditions of *T. thermophilus* $\alpha$ Expressed as a Translationally Coupled Protein

Lysis was accomplished by creation of spheroplasts of the cells carrying the expressed *T. thermophilus*  $\alpha$ -subunits. First, 50 g of a 1:1 suspension of frozen cells (25 g cells) in Tris-sucrose which had been stored at -20°C were added to 69 ml tris-sucrose that had been pre-warmed to 55°C (2.75 ml/g of cells). To the stirred mixture, 1.25 ml of 0.5 M 1,4-dithiothreitol (DTT) (0.05 ml/g of cells) and 6.25 ml of lysis buffer (2M NaCl, 0.3M spermidine in Tris-sucrose adjusted to pH 7.5) (0.25 ml/g of cells) was added. The presence of 18 mM spermidine kept the nucleoid condensed within partially disrupted cells and displaced DNA binding proteins. The pH of the slurry was adjusted to pH 8.0 by the addition of 0.5 ml of 2 M Tris base (pH is

- 83 -

adjusted to 8.0 with 2 M Tris base), and 125 mg lysozyme was added resuspended in 4.5 ml of Tris-sucrose buffer (5 mg lysozyme/g of cells). The slurry was distributed into 250 ml centrifuge bottles after stirring 5 min and incubated at 4°C for 1 hour. The 250 ml centrifuge bottles were then placed in a 37°C swirling water bath and gently inverted every 30 seconds for 4 minutes. The supernatant was separated from insoluble cellular debris by centrifugation (23,000 x g, 60 min, 4°C). The recovered supernatant (0.1 l) constituted Fraction I (Fr I) (13 mg protein/ml). All protein concentrations here and below are determined using the Coomassie Protein Assay Reagent from Pierce and bovine serum albumin (BSA) as a standard. FrI was divided into 5 equal volumes and 0.164, 0.226, 0.291, 0.361 and 0.436 g of ammonium sulfate (30%, 40%, 50%, 60% and 70% saturation) was added for each ml of FrI in the separate sample, respectively, over a 15 min interval at 4°C. The mixture was stirred for an additional 30 min at 4°C. The precipitate was collected by centrifugation (23,000 x g, 45 min, 0°C). The resulting pellets were resuspended in 2 ml Ni-NTA suspension buffer (50 mM Tris-HCl (pH 7.5), 40 mM KCl, 7 mM MgCl<sub>2</sub> and 10% glycerol. The protein concentration of each sample was determined using the Coomassie Protein Assay Reagent (Pierce) and bovine serum albumin (BSA) as a standard. The 30%, 40%, 50%, 60% and 70% ammonium sulfate precipitated samples contained protein concentrations of 2.4, 8.0, 18.0, 35.0 and 38.0 mg/ml, respectively (FIG. 3).

The samples were analyzed by SDS-polyacrylamide gel electrophoresis (FIG. 4). The 40% ammonium sulfate precipitated samples contained over 90% of the  $\alpha$ -subunit.

Each ammonium sulfate cut was also assayed for activity in gap-filling assays describe above in the section entitled "Gap Filling Assay for Determination of *T. thermophilus*  $\alpha$ -subunit Activity". The activity appears to be highest at 40% ammonium sulfate saturation and drops as percent ammonium sulfate saturation increased (FIG. 5). This is due to either higher salt being retained in the resuspended pellet and effecting the gap filling



- 84 -

reaction, or an inhibiting contaminant precipitating at the higher ammonium sulfate concentrations and effecting activity of the *T. thermophilus*  $\alpha$ -subunit. Since SDS-polyacrylamide gel electrophoresis and activity assays indicate that most of the  $\alpha$ -subunit is being recovered in 40% ammonium sulfate cuts, this concentration of ammonium sulfate was used in subsequent preparations.

#### Purification of *T. thermophilus dnaE* Product ( $\alpha$ -subunit) from pTAC-CCA-TE

Lysis was accomplished by creation of spheroplasts of cells carrying the expressed *T. thermophilus*  $\alpha$  (large-scale preparation of 7-10-2000). First, 500 g of a 1:1 suspension of frozen cells (250 g cells) in Tris-sucrose stored at  $-20^{\circ}\text{C}$  were used to prepare Fr I (770 ml, 27.4 mg/ml). The preparation was as described in the section entitled "Determination of Optimal Ammonium Sulfate Precipitation Conditions of *T. thermophilus*  $\alpha$ -subunit Expressed as a Translationally Coupled Protein". To Fr I, ammonium sulfate (0.258 g to each initial ml Fraction I-45% saturation) was added over a 15 min interval. The mixture was stirred for an additional 30 min at  $4^{\circ}\text{C}$  and the precipitate was collected by centrifugation (23,000 x g, 45 min,  $0^{\circ}\text{C}$ ). The resulting pellets were quick frozen by immersion in liquid nitrogen and stored at  $-80^{\circ}\text{C}$ .

The pellets from Fr I were resuspended in 160 ml of 50 mM Tris-HCl, (pH 7.5), 25% glycerol, 1 mM EDTA, 1 mM DTT and homogenized using a Dounce homogenizer. The sample was clarified by centrifugation (16,000 x g) and the supernatant constituted Fr II (164 ml, 11.4 mg/ml). Fr II was further purified using a Butyl Sepharose Fast Flow (Pharmacia Biotech) column. The butyl resin (360 ml) was equilibrated in butyl equilibration buffer (50 mM Tris-HCl, (pH 7.5), 25% glycerol, 1 mM EDTA, 1 mM DTT, 0.5 M ammonium sulfate). The column was poured using 250 ml of Butyl resin. The remaining 110 ml of Butyl resin was mixed with Fr II giving 274 ml. To this mixture, 0.5 volume of saturated ammonium sulfate was added slowly while stirring over a period of 1 hour. This mixture was added to the column at 1.3 ml/min. The column was then washed with 1 L of equilibration buffer. The

- 85 -

protein was eluted in 10 column volumes of a gradient beginning with butyl equilibration buffer and ending in a buffer containing 50 mM Tris-HCl, (pH 7.5), 25 % glycerol, 1 mM EDTA, 1 mM DTT, 50 mM KCl. Remaining protein was removed from the column by eluting with an additional 10 column  
5 volumes "bump" of the end buffer. The  $\alpha$ -subunit eluted in the first half of the "bump", and was pooled (242 ml, 0.15 mg/ml). The gap-filling assay was used to assay fractions for activity.

The pool was concentrated to 27 ml (1.5 mg/ml) using polyethylene glycol (PEG) 8000 in powder form (Fisher). *T. thermophilus*  $\alpha$  was further  
10 purified using a Sephacryl S300 HR (Pharmacia Biotech) gel filtration column (510 ml, 3 cm x 120 cm) equilibrated in 50 mM Tris-HCl, (pH 7.5), 20 % glycerol, 100 mM NaCl, 1 mM EDTA, 5 mM DTT. The column was loaded and the protein eluted at a flow rate of 0.7 ml/min. The  $\alpha$ -subunit was isolated as a highly purified protein (35 ml, 0.23 mg/ml). A 10% SDS-polyacrylamide  
15 gel summarized the stages of purification of native *T. thermophilus*  $\alpha$  (FIG. 6).

### EXAMPLE 3

Construction of pA1-NB-TX that Expresses *T. thermophilus dnaX* ( $\tau$  and  $\gamma$ -subunits) Fused to an N-Terminal Peptide That Contains Hexahistidine and a  
20 Biotinylation Site

The *T. thermophilus dnaX* gene was previously inserted into pA1-CB-ClaI to be expressed as both native (pA1-TX) and C-terminal tagged proteins (pA1-CB-TX) (U.S. Application No. 09/151,888). Both  $\tau$  and  $\gamma$  subunits  
25 were expressed at low levels from both constructs. The *T. thermophilus dnaX* gene was also previously inserted into pET-CB-ClaI plasmids to be expressed as both native (pET-TE) and C-terminal tagged proteins (pET-CB-TX) (U.S. Application 09/151,888). As when under control of the pA1 promoter, when expressed under control of the T7 promoter, both  $\tau$  and  $\gamma$ -subunits were  
30 express at low levels. In an attempt to increase expression levels of  $\tau$  and  $\gamma$  subunits, plasmids were designed to fuse the *dnaX* gene to DNA encoding an N-terminal peptide that contains hexahistidine and a biotinylation site (ATG

- 86 -

project S). First, a PCR reaction was designed to amplify a fragment of the N-terminus (5') of the *dnaX* gene from the plasmid pA1-TX. The forward (ATG primer #P38-S1586, 5'-AACTGCAGAGCGCCCTCTACCG-3') (SEQ ID NO:47) adds a *Pst*I site to the 5' end of the *dnaX* gene so that the actual PCR product excludes the ATG start codon and begins at codon 2. The *Pst*I restriction site adjacent to codon 2 brings the 5' portion of the *dnaX* gene in frame with the N-terminal fusion peptide coding sequences. The reverse primer (ATG primer #P38-A2050, 5'-CGGTGGTGGCGAAGACGAAGAG-3') (SEQ ID NO:48) was designed so that it is downstream of the *Bam*HI restriction site within *T. thermophilus dnaX* (the *Bam*HI restriction site is approximately 318 bases downstream of the start codon). This PCR product was cut with *Pst*I and *Bam*HI and ligated into pAI-NB-AgeI that had been cut with the same two restriction enzymes. This plasmid was transformed into DH5 $\alpha$  and positive isolates were selected by ampicillin-resistance. Plasmids from one positive clone were verified by *Bam*HI/*Pst*I restriction digest (yielding the expected 5.5 kb and 0.32 kb fragments) and *Nco*I digest (yielding the expected 5.6 and 0.16 kb fragments). The sequence of the inserted region was confirmed by DNA sequencing (ATG SEQ #1185 and 1186, primers P64-S10 and P64-A215) and compared to the sequence of pA1-TX. This precursor plasmid was named pAI-NB-TX5' and the isolate (pAI-NB-TX5'/ DH5 $\alpha$ ) was stored as a stock culture (ATG glycerol stock #702).

Next, the 3' region (C-terminus) of the *dnaX* gene (1.6 kb) was cut out of the pAI-TX plasmid using the restriction enzymes *Bam*HI and *Spe*I. This fragment was ligated into the precursor plasmid pAI-NB TX5' that has been cut with the same two restriction enzymes. This plasmid was transformed into DH5 $\alpha$  and plasmid containing colonies were selected by ampicillin-resistance. Positive isolates were verified by *Bam*HI/*Spe*I digest yielding the expected 5.9 and 1.6 kb fragments. This plasmid containing the entire gene for TX linked to the N-terminal fusion peptide was named pAI-NB-TX and the isolate (pAI-NB-TX/ DH5 $\alpha$ ) was stored as a stock culture (ATG glycerol stock #740).

- 87 -

Verification of Expression of *T. thermophilus dnaX* gene ( $\tau$  and  $\gamma$ -subunits)  
Fused to an N-Terminal Peptide That Contains Hexahistidine and a  
Biotinylation Site by pA1-NB-TX/AP1.L1

5           The pA1-NB-TX plasmid was prepared and transformed into both  
MGC1030 (ATG glycerol stock #740) and AP1.L1 bacteria (ATG glycerol  
stock #741). The bacterial growth and isolation of total protein was as  
described in Example 2. An aliquot of supernatant (3  $\mu$ l) containing total  
10       protein was loaded onto a 4-20% SDS-polyacrylamide mini-gel (Novex,  
EC60255; 1 mm thick, with 15 wells/gel) in 25 mM in Tris base, 192 mM  
glycine, and 0.1% SDS. The mini-gel was stained with Coomassie Blue, and  
one protein (doublet band) was observed to be migrating below 60 kDa and  
the other protein band slightly above the 60 kDa molecular weight standards,  
of the Gibco 10 kDa protein ladder. These protein bands were observed as  
15       distinct bands in the induced cultures from both bacterial strains, but was not  
observed in the uninduced controls. These proteins were determined to be  
consistent with the expected molecular weights of 53.6 and 61.9 kDa. The  
detected proteins bands representing *T. thermophilus* DnaX represented less  
than 2% of the total *E. coli* protein, based on the intensity of Coomassie Blue  
20       staining of the protein bands on the gel.

In *T. thermophilus*, the putative frameshift site, allowing expression of  
both ( $\tau$  and  $\gamma$ -subunits, has the sequence A AAA AAA A, which would enable  
either a +1 or -1 frameshift. The +1 frameshift product would extend only  
one residue beyond the lys-lys encoding sequence, similar to the *E. coli* -1  
25       frameshift product. However, the -1 frameshift would encode a protein with a  
12-amino acid extension. This would allow the expression of two  $\gamma$ -subunits  
differing in size by 11 amino acids. Alternatively, recent work has indicated  
that the *T. thermophilus*  $\gamma$ -subunit may be expressed as the result of  
transcriptional slippage producing a sub-population of different length  
30       mRNAs encoding two different length gamma subunits (Larsen, B., Wills, *et*  
*al.*, *Proc. Natl. Acad. Sci.* 97:1683-1688 (2000)). We observe  $\gamma$ -subunit as a  
doublet protein band, confirming that one of these processes is occurring.

- 88 -

Next, the expressed proteins were subjected to biotin blot analysis as described in Example 2. The endogenous *E. coli* biotin-CCP protein, ~20 kDa was detectable in both induced and non-induced samples. Two bands of equal intensity were visualized, one just below 60 kDa and the other slightly above 60 kDa molecular weight standards, of the Gibco 10 kDa protein ladder in the induced cultures from both bacterial strains, but was not observed in the uninduced control.

#### Optimization of Expression of *T. thermophilus* DnaX by pA1-NB-TX

Since expression of *T. thermophilus dnaX* gene yielded low or no detectable proteins when expressed as both a native or coupled to an C-terminal fusion peptide, extra care was taken with *dnaX* linked to an N-terminal fusion peptide to achieve optimum expression. Expression was analyzed using both *E. coli* strains MGC1030 and AP1.L1 carrying pA1-NB-TX at different induction times and also at different growth temperatures (25 and 37°C). Growth of bacterial cultures and analysis were carried out as described in Example 2. Biotin blot analysis indicated that expression levels were higher at 37°C and also slightly better when expressed in the AP1.L1 bacterial strain (FIG. 7). The optimum yield of *T. thermophilus* DnaX was attained by 4 h post induction and at 37°C; this induction time will be used in subsequent experiments.

#### Large Scale Growth of pA1-NB-TX/AP1.L1

Strain pA1-NB-TX/AP1.L1 was grown in a 250 L fermentor (fermentation run #99-17), to produce cells for purification of *T. thermophilus dnaX* ( $\tau$  and  $\gamma$ -subunits) fused to an N-terminal peptide that contains hexahistidine and biotinylation site as described Example 2. Cell harvest was initiated 4 hours after induction at OD<sub>600</sub> = 7.0, and the cells were chilled to 10°C during harvest. The harvest volume was 172 L, and the final harvest

- 89 -

weight was approximately 2.2 kg of cell paste. An equal amount (w/w) of 50 mM Tris-HCl (pH 7.5) and 10% sucrose solution was used to resuspend the cell paste. Cells were frozen by pouring the cell suspension into liquid nitrogen, and stored at  $-20^{\circ}\text{C}$  until processed. Quality control results showed 10 out of 10 positive colonies on ampicillin-containing medium in the inoculum and 10 out of 10 positive colonies at harvest.

Purification of *T. thermophilus dnaX* Product (( $\tau$  and  $\gamma$ -subunits) Fused to an N-Terminal Peptide That Contains Hexahistidine and a Biotinylation Site

Lysis of 800 g of a 1:1 suspension of frozen cells (400 g of cells) containing pA1-NB-TX stored in Tris-sucrose at  $-20^{\circ}\text{C}$ , was preformed as described in Example 2. The recovered supernatant (1.75 l) constituted Fraction I (Fr I) (13.5 mg/ml). To Fr I, ammonium sulfate (0.226 g to each initial ml Fraction I-40% saturation) was added over a 15 min interval. The mixture was stirred for an additional 30 min at  $4^{\circ}\text{C}$  and the precipitate was collected by centrifugation (23,000 x g, 45 min,  $0^{\circ}\text{C}$ ). The resulting pellets were quick frozen by immersion in liquid nitrogen and stored at  $-80^{\circ}\text{C}$ .

The pellets from Fr I were resuspended in 125 ml of  $\text{Ni}^{++}$ -NTA suspension buffer (50 mM Tris-HCl (pH 7.5), 40 mM KCl, 7 mM  $\text{MgCl}_2$ , 10% glycerol, 7 mM  $\beta\text{ME}$ , 0.1 mM PMSF) and homogenized using a Dounce homogenizer. The sample was clarified by centrifugation (16,000 x g) and the supernatant constituted Fr II (13.3 mg/ml). Fr II was added to 60 ml of a 50% slurry of Ni-NTA resin in  $\text{Ni}^{++}$ -NTA suspension buffer and rocked for 1.5 hours at  $4^{\circ}\text{C}$ . This slurry was then loaded onto a BioRad Econo-column (2.5 x 5 cm). The column was washed with 300 ml of  $\text{Ni}^{++}$ -NTA wash buffer (50 mM Tris-HCl (pH 7.5), 1 M KCl, 7 mM  $\text{MgCl}_2$ , 10% glycerol, 10 mM Imidazole, 7 mM  $\beta\text{ME}$ ) at a flow rate of 0.5 ml/min. The NB-TX protein was eluted in 300 ml of  $\text{Ni}^{++}$ -NTA elution buffer (50 mM Tris-HCl (pH 7.5), 40 mM KCl, 7 mM  $\text{MgCl}_2$ , 10% glycerol, 7 mM  $\beta\text{ME}$ ) containing a 10-200 mM imidazole-HCl (pH 7.5) gradient. The eluate was collected in 150 x 2 ml fractions. The protein concentration of each fraction was determined (FIG. 8).

- 90 -

Fractions were analyzed by SDS-polyacrylamide gel electrophoresis, (FIGs. 9A and 9B) and observed to contain only one major higher molecular weight contaminant. This contaminant migrated just above the  $\tau$ -subunit and disappeared by fraction 96. Fractions 66-95 and 96-113 were pooled and the proteins were precipitated by addition of ammonium sulfate (0.226 g to each initial ml Fraction I-40% saturation). The precipitate was collected by centrifugation (23,000 x g, 45 min, 0°C) and stored at -80°C. A portion of N-terminal tagged *T. thermophilus* DnaX purified using Ni<sup>++</sup>-NTA column chromatography was stored as laboratory stocks.

In an additional purification step for antibody production, pellets containing ammonium sulfate precipitated N-terminal tagged *T. thermophilus* DnaX were resuspended in 30ml of phosphate buffered saline (PBS) (137 mM NaCl, 2.7 mM KCl, 4.3 mM Na<sub>2</sub>HPO<sub>4</sub>·7H<sub>2</sub>O, 1.4 mM KH<sub>2</sub>PO<sub>4</sub> (pH 7.3)) plus 10% glycerol and homogenized using a Dounce 75 homogenizer. The resulting solution was clarified by centrifugation (16,000 x g) and the supernatant constituted Fr III (2.9 mg/ml).

Fr III was loaded onto a 2 ml UltraLink<sup>TM</sup> Immobilized Monomeric Avidin column (1.1 cm x 2.5 cm) (Pierce) equilibrated in PBS plus 10% glycerol as per manufacturer instructions. The sample was loaded at a flow rate of 0.09 ml/min. The flow through was passed back through the column three times to allow all biotinylated protein to bind the avidin. The column was next washed with 10 ml PBS plus 10% glycerol at a flow rate of 0.08 ml/min. The protein was eluted from the column in 20 ml of elution buffer (2 mM D-biotin, 10% glycerol in PBS) at a flow rate of 0.09 ml/min (FIGs. 10A and 10B).

This purification step removed the upper molecular weight contaminant observed in the Ni<sup>++</sup>-NTA column purification. Fractions 1-24 (19 ml) were pooled (0.43 mg/ml) and the protein was precipitated by addition of ammonium sulfate (0.258 g to each ml of pooled fractions) and centrifuged as described above and stored at -80°C. This sample was used in production of polyclonal antibodies described below.

- 91 -

Production of polyclonal antibodies against *T. thermophilus* DnaX ( $\tau$  and  $\gamma$ -subunits)

5 For production of polyclonal antibodies, the pellets containing N-tagged *T. thermophilus* DnaX from the avidin purification were dissolved in 2 ml of PBS and dialyzed against 500 ml of PBS two times (2.5 mg/ml, 2 ml). The sample was diluted to 50  $\mu$ g/ml in PBS and 2 ml was injected directly into a vial containing adjuvant (RIBI Adjuvant System (RAS)). This solution was  
10 mixed and allowed to come to room temperature. One ml of the adjuvant/NB-TX mixture was used to inoculate a rabbit (#598); 0.05 ml in each of six sites intradermal injections, 0.3 ml intramuscular injections in each hind leg, and 0.1 ml subcutaneous injection in the neck region. Before the initial injection a 5 ml preinjection bleed was collected. The rabbit received a booster using  
15 one-half the initial injection volume 28 days post initial inoculation. A test bleed (10 ml) was collected on day 37. The rabbit received a second booster using the same formulation as original inoculation at day 58. Total blood was collected on day 72.

20 The optimum dilutions of anti-serum for binding NB-TX was determined after the test bleed and after the final bleed. This was carried out using SDS-polyacrylamide gel electrophoresis in which a small aliquot of *T. thermophilus* N-terminal tagged DnaX (1.0  $\mu$ g/well) was electrophoresed onto a 10% SDS-polyacrylamide mini-gel (10 x 10 cm), and then the protein was transferred onto nitrocellulose membrane. The membrane was cut into strips  
25 with each strip containing an identical band of *T. thermophilus* N-terminal tagged DnaX. The membrane was blocked in 0.2% Tween 20 (v/v)-TBS (TBST) containing 5% non-fat dry milk (w/v) for 1 hour at room temperature, rinsed with TBST. The strips were placed in antiserum/TBST (dilutions of; 1:100, 1:200, 1:400, 1:800, 1:1600, 1:3200, 1:6400, and 1:12800) for 1 hour  
30 and then washed 4 times for 5 min in TBST. Next, the strips were placed in secondary antibody-conjugated to alkaline phosphatase (goat anti-rabbit IgG (H+L), 1:3000 dilution in TBST) (BioRad) for 1 hour. The strips were then



- 92 -

washed 4 times for 5 min with TBST. Following this extensive washing, the blots were developed with BCIP/NBT (KPL #50-81-07; one component system). Proteins corresponding to the  $\tau$  and  $\gamma$ -subunits were visualized as distinct bands even at the highest dilution of antiserum. These bands became more intense as the dilution of antiserum was decreased. The negative control contained antiserum taken from the rabbit prior to inoculating with antigen. The positive control is a biotin blot analysis of the antigen at the same concentration (1.0  $\mu$ g) as used in antiserum detection (FIG. 11).

Next, the minimum amount of *T. thermophilus* N-terminal tagged DnaX needed for recognition by antibody serum was determined. This was carried out using SDS-polyacrylamide gel electrophoresis in which small aliquots of *T. thermophilus* N-terminal tagged DnaX (0.02, 0.04, 0.08, 0.16, 0.32, 0.625, 1.25, 2.50, and 5.0  $\mu$ g/well) were electrophoresed onto a 10% SDS-polyacrylamide mini-gel (10 x 10 cm). The protein was transferred onto nitrocellulose membrane. The blotted nitrocellulose was blocked in TBST containing 5% non-fat dry milk (w/v) for 1 hour at room temperature, rinsed with TBST. The blot were placed in antiserum/TBST (dilution of 1:6400) for 1 hour and then washed 4 times for 5 min in TBST. Next, the blot was placed in secondary antibody-conjugated to alkaline phosphatase (goat anti-rabbit IgG (H+L), 1:3000 dilution in TBST) (BioRad) for 1 hour. The blot was then washed 4 times for 5 min with TBST. Following this extensive washing, the blot was developed with BCIP/NBT (KPL #50-81-07; one component system) (FIG. 12).

Proteins corresponding to  $\tau$  and  $\gamma$  were visualized as distinct bands at 0.02  $\mu$ g of DnaX. These bands became more intense as the concentration of DnaX was increased (FIG. 12).

Production of monoclonal antibodies against *T. thermophilus dnaX* ( $\tau$  and  $\gamma$ -subunits)

Two ml of the sample of *T. thermophilus* DnaX was diluted to 50  $\mu$ g/ml in PBS (described above) was injected directly into a vial containing

- 93 -

adjuvant (RIBI Adjuvant System (RAS)). On day 0, three mice were inoculated with the DnaX-adjuvant sample (0.2 ml/mouse). At day 21, each mouse received a booster of 0.2 ml of the DnaX-adjuvant sample. On day 41, a test bleed was collected from tail clippings. The three mice were boosted a second time on day 44, and a second bleed from tail clippings was collected on day 58. Antiserum from this bleed was used for Western analysis as described in the section entitled "Production of polyclonal antibodies against *T. thermophilus dnaX* ( $\tau$  and  $\gamma$ -subunits)". The antiserum was used at a 1:400 dilution to detect 1 $\mu$ g/lane of *T. thermophilus* DnaX. The antiserum was also used in ELISA screening (Tissue Culture/Monoclonal Antibody Facility, UCHSC). Mouse #2 and #3 gave equal response to *T. thermophilus* DnaX in both Western analysis and ELISA screening, while mouse #1 gave a lower response. Mouse #2 was selected and given to the Tissue Culture/Monoclonal Antibody Facility (UCHSC) for production of mono-clonal antibodies against N- terminal tagged *T. thermophilus* DnaX.

Cloning *T. thermophilus dnaX* gene ( $\tau/\gamma$ ) into a translationally coupled vector pTAC-CCA-ClaI

To efficiently express  $\tau/\gamma$  as a native protein a vector was designed to express  $\tau/\gamma$  as a translationally coupled proteins. The goal here is again to use translational coupling as described Example #2. The *dnaX* gene was inserted behind the CCA adding enzyme and translationally coupled as described for native *T. thermophilus*  $\alpha$ . First, the *dnaX* gene was amplified by using pA1-TX as a template by PCR. The forward/sense primer (ATG primer #P38-S1cla2, 5'-ACTTATCGATAATGAGCGCCCTCTACCGCC-3') (SEQ ID NO:49) has a *ClaI* restriction site in the non-complementary region. The non-complementary region also contains the "TA" of the stop (TAA) for the upstream CCA-adding protein fragment. The region of the primer complementary to the 5' end of the *T. thermophilus holA* gene begins with "A" which is the first nucleotide of the "ATG" start codon and the final "A" of the "TAA" stop codon. The reverse/antisense primer (ATG primer #P38-

- 94 -

A1603STOP<sub>spe</sub>, 5'-GAGGACTAGTTTATTATATACCAGTACCCCCT  
ATC-3') (SEQ ID NO:50) contains a *Spe*I restriction site in the non-  
complementary portion of the primer and also an additional stop codon  
adjacent to the native stop codon, giving two stop codons in tandem. There  
was also a clamp region for efficient cutting with *Spe*I. Next, the PCR product  
was digested with *Cla*I/*Spe*I restriction enzymes and inserted into the pTAC-  
CCA-*Cla*I plasmid digested with the same enzymes. The plasmid was  
transformed into DH5 $\alpha$  bacteria and plasmids from ampicillin-resistant  
positive isolates were screened for by digestion with *Cla*I/*Spe*I restriction  
enzymes yielding 1.6 and 5.5 kb fragments. The sequence of both strands of  
the insert were verified by DNA sequencing (ATG SEQ #1666-1674, 1617,  
1719; primers, P144-S23, P144-A1965, P38-S394, P38-S809, P38-S1169,  
P38-A1272, P38-A946, P38-A541, P38-A282, P38-A106). Sequence analysis  
confirmed the correct sequence was contained within the inserted region. This  
plasmid was named pTAC-CCA-TX and the isolate was stored as a stock  
culture (ATG glycerol stock #1030).

Verification of expression of native *T. thermophilus* DnaX proteins by PTAC-  
CCA-TX/MGC1030 and pTAC-CCA-TX/AP1.L1

The pTAC-CCA-TX plasmid was prepared and transformed into  
MGC1030 bacteria (ATG glycerol stock #1067, 1068, 1069) and AP1.L1  
(ATG glycerol stock #1075, 1076, 1077). The bacterial growths and isolation  
of total cellular protein were as described in Example 2. A small aliquot of  
each supernatant (3  $\mu$ l) containing total cellular protein was electrophoresed  
onto a 4-20% SDS-polyacrylamide mini-gel (Novex, EC60255; 1 mm thick,  
with 15 wells/gel) in 25 mM in Tris base, 192 mM glycine, and 0.1% SDS.  
The mini-gels were stained with Coomassie Blue. The region of the gels  
expected to contain  $\tau$  (58.3 kDa) or  $\gamma$  (51.0 kDa) contained many native *E. coli*  
proteins and  $\tau$  or  $\gamma$  could not be visualized in any of the isolates.

- 95 -

## EXAMPLE 4

Identification of *T. thermophilus* *holA* gene ( $\delta$ -subunit)

The sequences of  $\delta$ -subunits from *E. coli* and *Haemophilus influenzae* and putative  $\delta$ -subunit sequences from *Bacillus subtilis*, *Aquiflex aeolicus* were used to search the *T. thermophilus* genome database at Goettingen Genomics Laboratory. A partial crude sequence of a region of the *T. thermophilus* genome containing a putative *T. thermophilus* *holA* gene was identified (using BLAST) and obtained (from Dr. Carsten Jacobi, Goettingen Genomics Laboratory, Institute of Microbiology and Genetics, Grisebachstrasse 8, Goettingen, Germany). There appeared to be several possible start sites that were all ATG and also a number of possible stop codons. Unsure of the accuracy of the crude sequence, the region of the *T. thermophilus* genome suspected of containing the *T. thermophilus* *holA* gene and flanking regions were amplified by PCR. PCR primers were designed using sequences derived from the crude sequence. The forward/sense primer (ATG primer P134-S415, 5'-CGGGAGGGTGAAGCGCAAGATGTC-3') (SEQ ID NO:51) and reverse/antisense primer (ATG primer P134-A2099, 5'-GCCGCACCCCCGCCCCGTAGT-3') (SEQ ID NO:52) using *T. thermophilus* genomic DNA as a template yielded a PCR product 1685 bp in length which contained the region of DNA encoding *holA*. This PCR fragment was inserted into pGEM-T Easy<sup>TM</sup> (Promega) vector per directions furnished by the manufacturer. The pGEM-T Easy<sup>TM</sup> Vector Systems takes advantage of the template independent addition of a single deoxyadenosine onto the 3'-end of PCR products by some thermostable DNA polymerases. The PCR fragments were ligated to linearized vector DNA that had been cleaved at the *EcoRV* site and had a single 3'-terminal thymidine added to both ends. By using these vectors, PCR products can be directly cloned without further enzymatic manipulation while taking advantage of the high efficiency of a cohesive-end ligation. This plasmid was transformed into DH5 $\alpha$  bacteria and positive isolates were selected by ampicillin-resistance.

- 96 -

Plasmids from one positive clone were isolated and screened by digestion with *EcoRI* restriction digest yielding 1.7 and 3.1 kb fragments. The sequence of the inserted DNA region was confirmed by DNA sequencing (ATG SEQ #1336-1345; primers, SP6, T7, P134-S621, P134-S1016, P134-S1279, P134-S1633, P134-A1849, P134-A1464, P134-A1091 and P134-A655). Numerous base changes were observed in the PCR clone compared to the crude sequence obtained from Goettingen Genomics Laboratory. An 876 bp open reading frame (ORF) was identified in the region containing the putative *T. thermophilus* *holA* gene. This isolate was stored as a stock culture (ATG glycerol stock #787).

The ORF identified in the PCR product above was amplified by PCR with *T. thermophilus* genomic DNA as a template. The forward/sense primer (ATG primer #P134-S585de).

5'-GGATCCAAGCTTCATATGGTCATCGCCTTCAC-3') (SEQ ID NO:53) contained a region complementary to the 5' end of the ORF. An *NdeI* site overlapped the ATG start codon, and there was also an upstream *HindIII* and *BamHI* site. The reverse/antisense primer (ATG primer #P134-A1493kpn, 5'-AGATCTGGTACCTCATCAACGGGCGAGGCGGAG-3') (SEQ ID NO:54) contained an additional stop codon adjacent to the native stop codon in the non-complementary region, giving two stop codons in tandem. There was a *KpnI* site upstream of the stop codons and a *BglII* restriction site upstream of the *KpnI* restriction site. This PCR fragment was inserted into pGEM-T Easy<sup>TM</sup> plasmids (Promega) as per manufacturer directions. The plasmid was then transformed into DH5 $\alpha$  bacteria and plasmids from ampicillin-resistant positive isolates were screened by *NdeI/KpnI* restriction digest yielding 0.9 and 3.0 kb fragments. The sequence of both DNA strands of the inserted region was confirmed by DNA sequencing (ATG SEQ #1392-1397, 1408; primers, SP6, T7, P134-S1279, P134-S1633, P134-A1464, P134-A790 and P134-A1849). This plasmid was named pT-TD(Kpn) and the isolate was stored as a stock culture (ATG glycerol stock #817).

- 97 -

The DNA coding sequence of the *T. thermophilus* *holA* gene (SEQ ID NO:9) is shown in FIG. 13. The start codon (atg) and the stop codon (tga) are in bold print. Also shown, in FIG. 14, is the protein (amino acid) sequence (SEQ ID NO:10) derived from the DNA coding sequence.

5        The amino acid sequence of *T. thermophilus*  $\delta$ -subunit was compared with the *E. coli*  $\delta$ -subunit (FIG. 15). Alignments were also made with all of the  $\delta$ -subunit sequences used in the *T. thermophilus* database search, *E. coli* and *Haemophilus influenzae* and putative  $\delta$ -subunit sequences from *Bacillus subtilis* and *Aquiflex aeolicus* (FIG. 16). The *T. thermophilus*  $\delta$ -subunit was  
10        34 %, 29%, 31% and 27% identical over a 193, 182, 110 and 169 amino acid overlap with *E. coli*, *H. influenzae*, *A. aeolicus* and *B. subtilis*  $\delta$ -subunits, respectively.

15        Construction of a Plasmid (pA1-NB-TD) that Overexpress *T. thermophilus* *holA* ( $\delta$ -subunit) Fused to an N-Terminal Peptide That Contains Hexahistidine and a Biotinylation Site

Since the  $\delta$ -subunit coupled to a C-terminal fusion peptide expressed poorly (described below), it was decided to attempt enhancement of  
20        expression by coupling the *holA* gene to an N-terminal fusion peptide. The *T. thermophilus* *holA* gene was inserted into the pA1-NB-Avr2 plasmid to be expressed fused to an N-terminal peptide containing hexahistidine and a biotinylation site. The *holA* gene was amplified by PCR using the pA1-TD plasmid as a template. The forward/sense primer adds a *Pst*I site to the 5'end  
25        of the gene so that the actual PCR product excludes the ATG start codon and begins at codon 2, with the *Pst*I site adjacent to codon 2 (ATG primer P134-S592pst, 5'-GAATTCTGCAGGTCATCGCCT TCACCG-3') (SEQ ID NO:11). The *Pst*I site will bring the *holA* gene into frame with the N-terminal fusion peptide and will add two amino acids (Leu and Gln) between the N-  
30        terminal fusion peptide and the second codon of the *holA* gene. The reverse primer was the same primer used in making pA1-TD (P134-A1493kpn). This primer was designed so two things could be accomplished. First, an additional

- 98 -

TGA (stop codon) was added to the end of the gene giving two stop codons in tandem (the natural stop codon and another one added in the non-complementary part of the primer). Second, a *KpnI* restriction site was added in the non-complementary region of the primer for insertion into the vector.

5 There was also a clamp region for efficient digestion with *KpnI*. The PCR product was digested with *PstI* and *KpnI* restriction enzymes and inserted into the pA1-NB-Avr2 plasmid digested with the same enzymes. The plasmid was transformed into DH5 $\alpha$  bacteria and plasmids from ampicillin-resistant positive isolates were screened for by digestion with *PstI* and *KpnI* restriction

10 enzymes yielding 0.9 and 5.62 kb fragments. This plasmid was selected and the sequence of both strands of the insert was verified by DNA sequencing (ATG SEQ #1530-1536; primers, P64-S10, P64-A215, P134-S1279, P134-S1633, P134-A1849, P134-A1464, P134-A790). This plasmid was named pA1-NB-TD and the isolate was stored as a stock culture (ATG glycerol stock

15 #915).

Verification of Expression of *T. thermophilus*  $\delta$ -subunit Fused to an N-Terminal Peptide That Contains Hexahistidine and a Biotinylation Site by pA1-NB-TD/MGC1030

20 The pA1-NB-TD plasmid was prepared and transformed into MGC1030 bacteria (ATG glycerol stock #931). The bacterial growths and isolation of total cellular protein were as described in Example 2. A small aliquot of each supernatant (3  $\mu$ l) containing total cellular protein was

25 electrophoresised onto a 4-20% SDS-polyacrylamide mini-gel (Novex, EC60255; 1 mm thick, with 15 wells/gel) in 25 mM in Tris base, 192 mM glycine, and 0.1% SDS. The mini-gels were stained with Coomassie Blue. A protein migrating just below the 40 kDa molecular weight standard of the

30 Gibco 10 kDa protein ladder could be detected as a distinct protein band, but was not observed in the uninduced control. This protein band corresponds to the expected molecular weight of the *T. thermophilus*  $\delta$ -subunit fused to the N-terminal fusion protein (36.2 kDa).

- 99 -

Next, the total protein in each lysate was transferred (blotted) from polyacrylamide gel to nitrocellulose as described in Example 2. Each lane contained 1.5 ul of the supernatant. The endogenous *E. coli* biotin-carboxyl carrier protein (biotin-CCP), ~20 kDa, was detectable in both induced and non-induced samples. A very intense protein band corresponding to the  $\delta$ -subunit migrated just below the 40 kDa molecular weight standards of the Gibco 10 kDa protein ladder. This protein was observed as a distinct band in the induced cultures, but was not observed in the uninduced control.

#### Optimization of Expression of *T. thermophilus* *holA* gene ( $\delta$ -subunit) by pA1-NB-TD

Expression was analyzed using the bacterial strains AP1.L1 carrying the pA1-NB-TD plasmid at different induction times. Bacterial growths and analysis were carried out as described Example 2. The growths and analysis were at 37°C. The total protein was analyzed using both SDS-polyacrylamide gel electrophoresis and biotin blot analysis (FIG. 17). Distinct protein bands corresponding to the  $\delta$ -subunit was observed by both forms of analysis. Biotin blot analysis indicates that most of the  $\delta$ -subunit is being expressed in 4 hours and at 37°C, these growth condition were used in subsequent preparations.

#### Large Scale Growth of *T. thermophilus* *holA* Gene Product ( $\delta$ -subunit) Fused to an N-Terminal Peptide That Contains Hexahistidine and Biotinylation Site by pA1-NB-TD/MGC1030

Strain pA1-NB-TD/MGC1030 was grown in a 250 L fermentor to produce cells for purification of *T. thermophilus*  $\delta$ -subunit as described in Example 2. Cell harvest was initiated 3 hours after induction, at OD<sub>600</sub> of 7.2, and the cells were chilled to 10°C during harvest. The harvest volume was 175 L, and the final harvest weight was approximately 2.47 kg of cell paste. An equal amount (w/w) of 50 mM Tris (pH 7.5) and 10% sucrose solution was added to the cell paste. Quality control results showed 10 out of 10 positive colonies on ampicillin-containing medium in the inoculum and 10/10 positive



- 100 -

colonies at harvest. Cells were frozen by pouring the cell suspension into liquid nitrogen, and stored at -20°C, until processed.

5 Determination of Optimal Ammonium Sulfate Precipitation Conditions of  $\delta$  Fused to an N-Terminal Peptide That Contains Hexahistidine and Biotinylation Site by pA1-NB-TD/MGC1030

Lysis was accomplished by creation of spheroplasts of the cells carrying the expressed *T. thermophilus*  $\delta$ -subunits. First, from 100 g of a 1:1  
10 suspension of frozen cells (50 g cells) in Tris-sucrose which had been stored at -20°C, FrI was prepared (160 ml, 23 mg/ml). The preparation was as described in Example 2. FrI was added to 2.4 ml of a 50% slurry of Ni-NTA resin equilibrated in Ni-NTA suspension buffer (50 mM Tris-HCl, (pH 7.5), 40 mM KCl, 7 mM MgCl<sub>2</sub>, 10 % glycerol, 7 mM  $\beta$ ME). The resin and sample  
15 were rocked for 1.5 hours at 4°C. The sample was then passed through a 5 ml fritted polypropylene column (Qiagen) to filter out the Ni-NTA resin and bound  $\delta$ . The resin was washed by passing 50 ml of Ni-NTA wash buffer through the column and eluted in 9 ml of Ni-NTA elution buffer (2.6 mg/ml).

The eluted sample was brought to 40 ml by added Ni-NTA suspension  
20 buffer. The sample was then divided into 4 equal volumes (10 ml) and 1.64, 2.26, 2.91 and 3.61 g of ammonium sulfate (30%, 40%, 50% and 60% saturation) was added to each separate sample, respectively, over a 15 min interval at 4°C. The mixture was stirred for an additional 30 min at 4°C and the precipitate was collected by centrifugation (23,000 x g, 45 min, 0°C). The  
25 resulting pellets were resuspended in 1 ml Ni-NTA suspension buffer. The protein concentration of each sample was determined using the Coomassie Protein Assay Reagent (Pierce) and bovine serum albumin (BSA) as a standard. The 30%, 40%, 50% and 60% ammonium sulfate precipitated samples contained protein concentrations of 0.4, 2.6, 3.2 and 3.5 mg/ml,  
30 respectively. The samples were analyzed by SDS-polyacrylamide gel electrophoresis (FIG. 18).

- 101 -

The 50% and 60% ammonium sulfate precipitated samples contained equal amounts of the  $\delta$ -subunit. The 40% ammonium sulfate precipitated samples contained approximately 90 % of that of the 50% and 60% ammonium sulfate precipitated samples, while the 30% ammonium sulfate precipitated sample contained very small amounts of the  $\delta$ -subunit. All future preparations of the  $\delta$ -subunit will be ammonium sulfate precipitated at 40% saturation.

Purification of *T. thermophilus* *holA* Product ( $\delta$ -subunit) Fused to an N-Terminal Peptide That Contains Hexahistidine and a Biotinylation Site by pA1-NB-TD/MGC1030

Lysis was accomplished by creation of spheroplasts of the cells carrying expressed *T. thermophilus*  $\delta$ . First, from 800 g of a 1:1 suspension of frozen cells (400 g cells) in Tris-sucrose which had been stored at  $-20^{\circ}\text{C}$ , FrI was prepared (1280 ml, 30.8 mg/ml). The preparation was as described Example 2. To Fr I, ammonium sulfate (0.226 g to each initial ml Fraction I-40% saturation) was added over a 15 min interval. The mixture stirred for an additional 30 min at  $4^{\circ}\text{C}$  and the precipitate was collected by centrifugation (23,000 x g, 45 min,  $0^{\circ}\text{C}$ ). The resulting pellets were quick frozen by immersion in liquid nitrogen and stored at  $-80^{\circ}\text{C}$ .

The pellets from Fr I were resuspended in 160 ml of  $\text{Ni}^{++}$ -NTA suspension buffer and homogenized using a Dounce homogenizer. The sample was clarified by centrifugation (16,000 x g) and the supernatant constituted Fr II (27.6 mg/ml). Fr II was added to 50 ml of a 50% slurry of Ni-NTA resin and rocked for 1.5 hours at  $4^{\circ}\text{C}$ . This slurry was then loaded onto a BioRad Econo-column (2.5 x 5 cm). The column was washed with 250 ml of  $\text{Ni}^{++}$ -NTA wash buffer at a flow rate of 0.5 ml/min. The protein was eluted in 230 ml of  $\text{Ni}^{++}$ -NTA elution buffer containing a 10-200 mM imidazole gradient. The eluate was collected in 92 x 2.5 ml fractions. Fractions were analyzed by SDS-polyacrylamide gel electrophoresis, and fractions 25-92 were found to contain  $\delta$  that was over 95% pure (FIGs. 19A

- 102 -

and 19B). Fractions 25-92 were pooled (160 ml, 2.3 mg/ml) and dialyzed against 3.5 L of HG.04 buffer (20 mM Hepes, (pH 7.6), 40 mM KCl, 1 mM MgCl<sub>2</sub>, 0.1 mM EDTA, 6 mM  $\beta$ ME, 10% glycerol). The dialyzed sample constituted Fr III (160 ml, 2.1 mg/ml). The sample was aliquoted, fast frozen in liquid nitrogen and stored at -80°C.

#### Production of polyclonal antibodies against *T. thermophilus* *holA* ( $\delta$ -subunits)

To recover ultra-pure protein for antibody production thirty ml of  $\delta$ -subunit Fr III from above (2.1 mg/ml) was precipitated using ammonium sulfate (7.75 g per initial ml of FrIII, 45% saturation). The precipitated pellets were resuspended in 20 ml PBS and purified using UltraLink™ Immobilized Monomeric Avidin column as described in Example 3. The protein elution profile of the avidin column is shown in FIG. 20. Fractions 2-6 (5 ml) were pooled (0.35 mg/ml). FIG. 21 shows the SDS-PAGE analysis of the Avidin column profile for *T. thermophilus*  $\delta$ .

The pooled samples were used to produce polyclonal antibodies against *T. thermophilus* *holA* gene product ( $\delta$ -subunit) as described in Example 3.

#### Construction of Plasmid (pA1-TD) that Overexpresses *T. thermophilus* *holA* gene ( $\delta$ -Subunit) as a Native Protein

Prior to construction of vector pA1-NB-TD to express  $\delta$  as an N-terminal tagged protein, several attempts were made to first express  $\delta$  as a native and a C-terminal tagged protein. These attempts were unsuccessful in producing adequate yields of  $\delta$  to justify purification attempts. These attempts are described in this section.

The *T. thermophilus* *holA* gene contained within the plasmid pT-TD(Kpn) was extracted by digestion of the plasmid with *NdeI*/*KpnI* restriction enzymes. This 0.9 kb fragment was inserted into pA1-CB-*NdeI*

- 103 -

which had been digested with the same restriction enzymes. The “ATG” of the *NdeI* site served as the start codon for the *holA* gene. This placed the start codon the correct distance (11 nucleotides) from the RBS for optimum translation. This plasmid was then transformed into DH5 $\alpha$  bacteria, and plasmids from ampicillin-resistant positive isolates were screened for by digestion with *NdeI* and *KpnI* restriction enzymes yielding 0.9 and 5.65 kb fragments. One plasmid was selected and the sequence of the insert verified by DNA sequencing (ATG SEQ #1428 and 1429, primers P38-S5576 and P134-S1633). This plasmid was named pA1-TD and the isolate was stored as a stock culture (ATG glycerol stock #841).

#### Verification of Expression of Plasmid (pA1-TD) that Overexpresses *T. thermophilus holA* Gene ( $\delta$ -Subunit) as a Native Protein from pA1-TD/MGC1030

Plasmid pA1-TD was prepared from DH5 $\alpha$  bacteria and transformed into MGC1030 bacteria (ATG glycerol stock #856, 857, 858). The bacterial growths of three isolates and isolation of total cellular protein were as described Example 2. A small aliquot (3  $\mu$ l) of supernatant containing total cellular protein from each of the three isolates was electrophoresed onto a 4-20% SDS-polyacrylamide mini-gel (Novex, EC60255; 1 mm thick, with 15 wells/gel) in 25 mM Tris base, 192 mM glycine, and 0.1% SDS. The mini-gel was stained with Coomassie Blue. There were no visible protein bands from any of the isolates corresponding to the predicted molecular weight of  $\delta$ .

#### Construction of a Plasmid (pA1-CB-TD) that Overexpresses *T. thermophilus holA* gene ( $\delta$ -subunit) Fused to a C-Terminal Peptide That Contains Hexahistidine and a Biotinylation Site

Again, since attempts to express the native  $\delta$ -subunit failed, we next tried to express this protein coupled to a C-terminal fusion peptide. The *holA* gene was amplified by PCR with *T. thermophilus* genomic DNA as a template. The forward/sense primer (ATG primer #P134-S585'de) was the same primer

- 104 -

used in construction of named pT-TD(Kpn) and contained a region complementary to the 5' end of the gene. Also, a *NdeI* site overlapped the ATG start codon, and there was also an upstream *HindIII* and *BamHI* site. The reverse/antisense primer was complementary to the 3' end of the ORF  
5 excluding the stop codon (ATG primer #P134-A1486spe, 5'-GAGGACTAGTACGGGCGAGGCGGAGGACC-3') (SEQ ID NO:43). This primer contained a *SpeI* restriction site adjacent to the complementary region of the primer. The *SpeI* site allowed for the expressed protein to contain two additional amino acids (Thr and Ser) between the C-terminal amino acid of the  
10  $\delta$ -subunit and the C-terminal fusion peptide. This 901 bp PCR product was inserted into pGEM-T Easy<sup>TM</sup> as previously described in the section entitled "Identification of *T. thermophilus holA* gene ( $\delta$ -subunit)". This plasmid was transformed into DH5 $\alpha$  bacteria and plasmids from ampicillin-resistant positive isolates were screened for by digestion with *NdeI* and *KpnI* restriction  
15 enzymes yielding 0.9 and 3.0 kb fragments. Both strands of the insert were verified by DNA sequencing (ATG SEQ #1398-1403 and 1409-1411; primers, SP6, T7, P134-S1279, P134-S1633, P134-A1464, P134-A790, P134-S1279, P134-A1849). This plasmid was named pT-TD(Spe) and the isolate was stored as a stock culture (ATG glycerol stock #818).

20 Plasmid pT-TD(spe) was prepared and the *holA* gene was extracted by digestion with *NdeI* and *KpnI* restriction enzymes. This 0.9 kb fragment was inserted into the pA1-CB-*NdeI* plasmid digested with the same restriction enzymes. This plasmid was transformed into DH5 $\alpha$  bacteria and plasmids from ampicillin-resistant positive isolates were screened for by digestion with  
25 *NdeI* and *KpnI* restriction enzymes yielding 0.9 and 5.65 kb fragments. The sequence of the inserted DNA fragment was confirmed by DNA sequencing (ATG SEQ # 1430,1431; primers, P38-S5576 and P134-S1633). This plasmid was named pA1-CB-TD and the positive isolate was stored as a stock culture (ATG glycerol stock #842).

30

- 105 -

Verification of Expression of *T. thermophilus*  $\delta$ -subunit Fused to a C-Terminal Peptide That Contains Hexahistidine and a Biotinylation Site by pA1-CB-TD/MGC1030

5           The pA1-CB-TD plasmid was prepared and transformed into MGC1030 bacteria (ATG glycerol stock #859). The bacterial growths of three isolates and isolation of total cellular protein were as described Example 2. A small aliquot of each supernatant (3  $\mu$ l) containing total cellular protein from each isolate was electrophoresed onto a 4-20% SDS-polyacrylamide  
10 mini-gel (Novex, EC60255; 1 mm thick, with 15 wells/gel) in 25 mM Tris base, 192 mM glycine, and 0.1% SDS. The mini-gels were stained with Coomassie Blue. The region of the gel in which CB-TD was expected contained other intense protein bands and  $\delta$  could not be visualized.

15           Next, the total protein in each lysate was transferred (blotted) from polyacrylamide gel to nitrocellulose as described in Example 2. Each lane contained 1.5  $\mu$ l of the supernatant. The endogenous *E. coli* biotin-CCP, ~20 kDa was detectable in both induced and non-induced samples. A very faint protein band corresponding to  $\delta$  migrated just below the 40 kDa molecular weight standard of the Gibco 10 kDa protein ladder. The predicted molecular  
20 weight of  $\delta$  is 36.2 kDa. This protein was observed as a faint band in the induced cultures, but was not observed in the uninduced control in lysates. The intensity of the protein bands indicated  $\delta$  was being expressed at very low levels.

25           Cloning *T. thermophilus* *holA* gene ( $\delta$ ) into a translationally coupled vector pTAC-CCA-ClaI

30           To efficiently express  $\delta$  as a native protein we designed a vector to express  $\delta$  as a translationally coupled protein. As with expression of DnaX as a translationally coupled protein, our goal here is also to use translational coupling as described in the Example 2. The *holA* gene was inserted behind the CCA adding enzyme and translationally coupled in two steps. First, the

- 106 -

*holA* gene was amplified using pA1-TD as a template by PCR. The forward/sense primer (ATG primer #P134-S588cla2, 5'-ACTGATCGATAATGGTCATCGCCTTAC-3') (SEQ ID NO:55) has a *Cla*I restriction site in the non-complementary region. As in the cloning strategy developed for pTAC-CCA-TX, the non-complementary region also contains the "TA" of the stop (TAA) for the upstream CCA-adding protein fragment. The region of the primer complementary to the 5' end of the *T. thermophilus holA* gene begins with "A" which is the first nucleotide of the "ATG" start codon and the final "A" of the "TAA" stop codon. The reverse/antisense primer (ATG primer #P134-A1491stopspe, 5'-GAGGTACTAGTCATCAACGGGCGAGGCGGAGGA-3') (SEQ ID NO:56) contains a *Spe*I restriction site in the non-complementary portion of the primer and also an additional stop codon adjacent to the native stop codon, giving two stop codons in tandem. There was also a clamp region for efficient cutting with *Spe*I. Next, the PCR product was digested with *Cla*I/*Spe*I restriction enzymes and inserted into the pTAC-CCA-*Cla*I plasmid digested with the same enzymes. The plasmid was transformed into DH5 $\alpha$  bacteria and plasmids from ampicillin-resistant positive isolates were screened for by digestion with *Cla*I/*Spe*I restriction enzymes yielding 0.9 and 5.5 kb fragments. The sequence of both strands of the insert were verified by DNA sequencing (ATG SEQ #1675-1681; primers, P144-S23, P144-A1965, P65-A106, P134-S1279, P134-S1633, P134-A1849, P134-A1464, P134-A790). Sequence analysis confirmed that the correct sequence was contained within the inserted region. This plasmid was named pTAC-CCA-TD and the isolate was stored as a stock culture (ATG glycerol stock #1031).

Verification of expression of native *T. thermophilus*  $\delta$ -subunit by PTAC-CCA-TD/MGC1030 and pTAC-CCA-TD/AP1.L1

The pTAC-CCA-TD plasmid was prepared and transformed into MGC1030 bacteria (ATG glycerol stock #1070) and AP1.L1 (ATG glycerol stock #1078). The bacterial growths and isolation of total cellular protein were

- 107 -

as described in Example #2. A small aliquot of each supernatant (3  $\mu$ l) containing total cellular protein was electrophoresised onto a 4-20% SDS-polyacrylamide mini-gel (Novex, EC60255; 1 mm thick, with 15 wells/gel) in 25 mM Tris base, 192 mM glycine, and 0.1% SDS. The mini-gels were stained with Coomassie Blue. A protein band corresponding to the predicted molecular mass of *T. thermophilus*  $\delta$  (32.5 kDa) was visualized migrating mid-way between the 30 and 40 kDa molecular weight standard of the Gibco 10 kDa protein ladder.

#### Large Scale Growth of pA1-CCA-TD/AP1.L1

Strain pA1-CCA-TD/AP1.L1 was grown in a 250 L fermentor to produce cells for purification of native *T. thermophilus*  $\delta$  as described in Example #2. Optimum induction times were determined as described in Example #2. Cell harvest was initiated 3 hours after induction, at OD<sub>600</sub> of 3.38, and the cells were chilled to 10°C during harvest. The harvest volume was 180 L, and the final harvest weight was approximately 1.56 kg of cell paste. An equal amount (w/w) of 50 mM Tris (pH 7.5) and 10% sucrose solution was added to the cell paste. Quality control results showed 10 out of 10 positive colonies on ampicillin-containing medium in the inoculum and 10/10 positive colonies at induction and 10/10 positive colonies at harvest. Cells were frozen by pouring the cells suspension into liquid nitrogen, and stored at -20°C, until processed.

#### Purification of native *T. thermophilus* $\delta$ from pA1-CCA-TD

Lysis was accomplished by creation of spheroplasts of the cells carrying the expressed *T. thermophilus*  $\delta$  -subunits. First, from 300 g of a 1:1 suspension of frozen cells (150 g cells) in Tris-sucrose which had been stored at -20 °C, FrI was prepared (930 ml, 16.4 mg/ml). The preparation was as described in Example #2. To Fr I, ammonium sulfate (0.258 g to each initial



- 108 -

ml Fraction I-45% saturation) was added over a 15 min interval. The mixture stirred for an additional 30 min at 4 °C and the precipitate was collected by centrifugation (23,000 x g, 45 min, 0 °C). The resulting pellets were quick frozen by immersion in liquid nitrogen and stored at -80 °C.

5 In the following purification steps, fractions from purification columns were assayed using the reconstitution assay (described in Example 7) to determine fractions that contained activity and therefore the  $\delta$ -subunit. The first purification step was conducted by Q Sepharose High Performance (Amersham Pharmacia) column chromatography (200 ml, 5.5 x 13 cm). The  
10 Q Sepharose resin was equilibrated in Q-sepharose equilibration buffer (25 mM Tris-HCl, (pH 7.5), 10% glycerol, 1 mM EDTA, 1 mM DTT, 10 mM KCl). The pellets from Fr I was resuspended in Q-sepharose resuspension buffer (25 mM Tris-HCl, (pH 7.5), 10% glycerol, 1 mM EDTA, 1 mM DTT) and homogenized using a Dounce homogenizer and clarified by centrifugation  
15 (16,000 x g). The sample was diluted in Q-sepharose resuspension buffer until the conductivity reached that of the equilibrated column and constituted Fr II (2250 ml, 0.8 mg/ml). Fraction II contained  $3.5 \times 10^9$  units of activity at  $1.84 \times 10^6$  units/mg protein. The sample was loaded onto the column and washed with 5 column volumes of Q-sepharose equilibration buffer. The wash was  
20 collected in 17 ml fractions (50 fractions). Analysis of the flow through from the column load and the fractionated wash indicated that  $\delta$  was present in the flow through from the column load and the first fractions from the column wash. The flow through from the column load and fractions 1-13 of the column wash were pooled and constituted Fr III (2470 ml, 0.05 mg/ml). SDS-polyacrylamide gel analysis of the pool indicated that *T. thermophilus*  $\delta$  had  
25 been purified over 16 fold by this purification step and contained  $3.5 \times 10^9$  units of activity at  $3.2 \times 10^7$  units/mg protein.

Fr III was further purified using Macro Prep Methyl HIC Support (BioRad) column chromatography. The methyl resin (60 ml) was equilibrated  
30 in methyl equilibration buffer (50 mM Tris-HCl, (pH 7.5), 10% glycerol, 1 mM EDTA, 1 mM DTT, 1 M ammonium sulfate). The column was poured

- 109 -

using 40 ml of methyl resin. The remaining 20 ml of methyl resin was mixed with Fr III giving 2490 ml. To this mixture, saturated ammonium sulfate (0.5 sample volume) was added slowly while stirring over a 1 hour period. This mixture was added to the column and allowed to flow through the column by gravity. The column was then washed with 5 column volumes (300 ml) of methyl equilibration buffer. The protein was eluted in 10 column volumes of 50 mM Tris-HCl, (pH 7.5), 10 % glycerol, 1 mM EDTA, 1 mM DTT buffer containing a 0.9 to 0.1 M gradient of ammonium sulfate and collected in 7 ml fractions (80 fractions). Fractions 29-42 contained *T. thermophilus*  $\delta$  that was over 90% pure. These fractions were pooled and analyzed using reconstitution assays (see Example 7) and SDS-polyacrylamide gels. The pooled fractions (100 ml, 0.14 mg/ml) constituted Fr IV and contained  $1.7 \times 10^9$  units of activity at  $1.23 \times 10^8$  units/mg protein.

*T. thermophilus*  $\delta$  was further purified using a Sephacryl S300 HR (Pharmacia Biotech) gel filtration column (510 ml, 3 cm x 120 cm) equilibrated in 50 mM Tris-HCl, (pH 7.5), 20 % glycerol, 100 mM NaCl, 1 mM EDTA, 5 mM DTT. The volume of Fr IV was reduced using PEG 8000 to 35 ml (0.22 mg/ml,  $8.2 \times 10^8$  Units). The sample was loaded onto the Sephacryl S-300 column and the protein eluted at a flow rate of 0.7 ml/min. The  $\delta$ -subunit was isolated as a highly purified protein (24 ml, 0.2 mg/ml). The pooled fractions constituted Fr V and contained  $5.8 \times 10^8$  units of activity at  $1.23 \times 10^8$  units/mg protein.

#### EXAMPLE 5

##### Identification of *T. thermophilus* *holB* gene ( $\delta'$ -subunit)

The amino acid sequence of  $\delta'$  from *E. coli* was used to search the *T. thermophilus* genome database at Goettingen Genomics Laboratory. A partial crude sequence of a region of the *T. thermophilus* genome containing a putative *T. thermophilus* *holB* gene was identified (using BLAST) and obtained (from Dr. Carsten Jacobi, Goettingen Genomics Laboratory, Institute

- 110 -

of Microbiology and Genetics, Grisebachstrasse 8, Goettingen, Germany). Unsure of the accuracy of the crude sequence, the region of the *T. thermophilus* genome suspected of containing the *T. thermophilus* *holB* gene and flanking regions were amplified by PCR. Two sets of PCR primers were designed using sequences derived from the crude sequence to insure that the proper sequence was identified. The first PCR reaction (ATG primers P139-S181, 5'-GGGGGACCGGATCGCCTTCTA-3' (SEQ ID NO:12) and P139-A1082, 5'-GTACGCCCCACGGTCATGTCTCTAAGTCT AAG-3' (SEQ ID NO:13)) used *T. thermophilus* genomic DNA as a template and yielded a PCR product of 901 bp fragment. This PCR fragment was inserted into pGEM-T Easy™ (Promega) vector per manufacturers directions. This plasmid was transformed into DH5α bacteria and ampicillin-resistant positive isolates were screened for by plasmid digestion with *EcoRI* restriction digest yielding 0.9 and 3.0 kb fragments. The correct sequence of both strands of the DNA in the inserted region were identified by DNA sequencing across the inserted region (ATG SEQ #1363, 1365-1367, 1379-1380; primers, SP6, T7, P139-S651, P139-S321, P139-A681, P139-A287). Three base changes (deletions of a "C" at positions 845 and 849, and G>C change at position 681) were observed in the PCR clone compared to the crude sequence obtained from Goettingen Genomics Laboratory. The deletions caused a frameshift leading to a larger open reading frame (ORF) (804 bp) than was seen in the crude sequence. This plasmid was named pT-TD'-1 and the isolate was stored as a stock culture (ATG glycerol stock #811).

The second PCR reaction utilized primers placed farther out from the putative *holB* gene (ATG primers #P139-S91, 5'-CTCCCCCCTCGGTGCGGGCCCTGGTGAA-3' (SEQ ID NO:14) and #P139-A1407, 5'-CTCGGCGCTGTAGTGGATGACG-3' (SEQ ID NO:15)) and also used the *T. thermophilus* genomic DNA as a template and yielded a PCR product of 1361 bp fragment. This PCR fragment was also inserted into pGEM-T Easy™ (Promega) vector per manufacturer directions. This plasmid was also transformed into DH5α bacteria and ampicillin-resistant positive isolates were

- 111 -

screened for by plasmid digestion with *EcoRI* restriction digest yielding 1.3 and 3.0 kb fragments. The correct sequence of both strands of the DNA in the inserted region were identified by DNA sequencing (ATG SEQ #1368-1372, 1381-1383; primers, SP6, T7, P139-S651, P139-S321, P139-1042, P139-A681, P139-A287, P139-A1082). Other base changes were observed in the 3' non-translated region when compared to the crude sequence obtained from Goettingen Genomics Laboratory. This plasmid was named pT-TD'-2 and the isolate was stored as a stock culture (ATG glycerol stock #812).

The DNA coding sequence of the *T. thermophilus* *holB* gene (SEQ ID NO:16) is in FIG. 22. The start codon (atg) and the stop codon (tga) are in bold print. Also shown, in FIG. 23, is the protein (amino acid) sequence (SEQ ID NO:17) derived from the DNA coding sequence.

The amino acid sequence of *T. thermophilus*  $\delta'$  was compared with that of the *E. coli*  $\delta'$ -subunit (FIG. 24). Other sequence alignments were carried out with  $\delta'$  sequences from *Bacillus subtilis*, *E. coli*, and *Haemophilus influenzae*, *Rickettsia* and putative  $\delta'$  sequences from *Aquiflex aeolicus* (FIG. 25). The *T. thermophilus*  $\delta'$ -subunit was 30 %, 29%, 31%, 39% and 31% identical over a 163, 149, 229, 104 and 104 amino acid overlap with *Bacillus subtilis*, *E. coli*, and *Haemophilus influenzae*, *Rickettsia* and a putative  $\delta'$ -subunit sequences from *Aquiflex aeolicus*, respectively.

Construction of a Plasmid (pA1-NB-TD') that Overexpress *T. thermophilus* *holB* ( $\delta'$ ) Fused to an N-Terminal Peptide That Contains Hexahistidine and a Biotinylation Site

To enhance expression of the *T. thermophilus*  $\delta'$ -subunit, the *holB* gene was cloned into the pA1-NB-AgeI plasmid to be expressed fused to an N-terminal peptide containing hexahistidine and a biotinylation site. The *holB* gene was amplified by PCR using the pA1-TD' plasmid (described below) as a template. The forward/sense primer adds a *PstI* site to the 5' end of the gene so that the actual PCR product excludes the ATG start codon and begins at codon 2, with the *PstI* site adjacent to codon 2 (ATG primer P139-S254pst,

- 112 -

5'-GAATTCTGCAGGCTCTAC ACCCGGCTCACCC -3' (SEQ ID NO:18)).

The *Pst*I site will bring the *holB* gene into frame with the N-terminal fusion peptide and will add two amino acids (Leu and Gln) between the N-terminal fusion peptide and the second codon of the *holB* gene. The reverse primer

(ATG primer P139-A1081stopspe, 5'-  
GGACACTAGTTCATCATGTCTCTAAGTCTAA-3' (SEQ ID NO:19) was

complementary to the 3' end of the *holB* gene including the additional TGA (stop codon). Also, a *Spe*I restriction site was added in the non-

complementary region of the primer for insertion into the vector. There was

also a clamp region for efficient cutting with *Spe*I. The PCR product was

digested with *Pst*I/*Spe*I restriction enzymes and inserted into the pA1-NB-AgeI plasmid digested with the same enzymes. The plasmid was transformed

into DH5 $\alpha$  bacteria and plasmids from ampicillin-resistant positive isolates

were screened for by digestion with *Pst*I/*Spe*I restriction enzymes yielding 0.8

and 5.6 kb fragments. The sequence of both strands of the insert were verified

by DNA sequencing (ATG SEQ #1537-1541, 1543; primers, P64-S10, P64-A215, P139-S321, P139-S651, P139-A681, P64-A215). Sequence analysis

confirmed that the correct sequence was contained within the inserted region.

This plasmid was named pA1-NB-TD' and the isolate was stored as a stock

culture (ATG glycerol stock #913).

Verification of Expression of *T. thermophilus*  $\delta'$ -subunit Fused to an N-Terminal Peptide That Contains Hexahistidine and a Biotinylation Site by

pA1-NB-TD'/MGC1030

The pA1-NB-TD' plasmid was prepared and transformed into MGC1030 bacteria (ATG glycerol stock #930). The bacterial growths and

isolation of total cellular protein were as Example 2. A small aliquot of each supernatant (3  $\mu$ l) containing total cellular protein was electrophoresised onto

a 4-20% SDS-polyacrylamide mini-gel (Novex, EC60255; 1 mm thick, with 15 wells/gel) in 25 mM in Tris base, 192 mM glycine, and 0.1% SDS. The

mini-gels were stained with Coomassie Blue. A protein migrating just below

- 113 -

the 40 kDa molecular weight standard of the Gibco 10 kDa protein ladder could not be detected in the lysates.

Next, the total protein in each lysate was transferred (blotted) from polyacrylamide gel to nitrocellulose as described Example 2. The endogenous *E. coli* biotin-CCP, ~20 kDa, was detectable in both induced and non-induced samples. An intense protein band corresponding to *T. thermophilus*  $\delta'$  migrated midway between the 30 and 40 kDa molecular weight standards of the Gibco 10 kDa protein ladder. The predicted molecular weight of *T. thermophilus*  $\delta'$  is 33 kDa. This protein was observed as a distinct band in the induced cultures, but was not observed in the uninduced control in lysates.

Optimization of Expression of *T. thermophilus* *holB* gene ( $\delta'$ -subunit) by pA1-NB-TD'

Expression was analyzed using the bacterial strains AP1.L1 carrying the pA1-NB-TD' plasmid at different induction times. Bacterial growths and analysis were carried out as described Example 2. The growths and analysis were at 37°C. The total protein was analyzed using both SDS-polyacrylamide gel electrophoresis and biotin blot analysis (FIG. 26). Distinct protein bands corresponding to  $\delta'$  were observed by both forms of analysis. Biotin blot analysis indicated that most of the  $\delta'$ -subunit is being expressed in 4 hours and at 37°C, these growth condition were used in subsequent preparations.

Determination of Optimal Ammonium Sulfate Precipitation Conditions of the  $\delta'$ -subunit Fused to an N-Terminal Peptide That Contains Hexahistidine and Biotinylation Site by pA1-NB-TD'/MGC1030

Lysis was accomplished by creation of spheroplasts of the cells carrying the expressed *T. thermophilus*  $\delta'$ -subunits. First, from 76.4 g of a 1:1 suspension of frozen cells (38.2 g cells) in Tris-sucrose which had been stored at -20°C, FrII was prepared and purified using a Ni<sup>++</sup>-NTA column as described in Example 2. The eluted sample was brought to 40 ml by added

- 114 -

Ni-NTA suspension buffer. The sample was then divided into 4 equal volumes (10 ml) and 1.64, 2.26, 2.91 and 3.61 g of ammonium sulfate (30%, 40%, 50% and 60% saturation) was added to each separate sample, respectively, over a 15 min interval at 4°C. The mixture rested for an additional 30 min at 4°C and was then centrifuged at 23,000 x g for 45 min at 0°C. The resulting pellets were resuspended in 1 ml Ni-NTA suspension buffer. The 30%, 40%, 50% and 60% ammonium sulfate precipitated samples contained protein concentrations of 0.47, 0.55, 1.3 and 1.2 mg/ml, respectively. The samples were analyzed by SDS-polyacrylamide gel electrophoresis. All four ammonium sulfate precipitated samples contained equal amounts of the  $\delta'$ -subunit. All future preparations of the  $\delta'$ -subunit will be ammonium sulfate precipitated at 35% saturation.

Large Scale Growth of *T. thermophilus* *holB* Gene Product ( $\delta'$ -subunit) Fused to an N-Terminal Peptide That Contains Hexahistidine and Biotinylation Site by pA1-NB-TD'/MGC1030

Strain pA1-NB-TD'/MGC1030 was grown in a 250 L fermentor, to produce cells for purification of *T. thermophilus*  $\delta'$ -subunit as described in Example 2. Cell harvest was initiated 3 hours after induction, at OD<sub>600</sub> of 5.8, and the cells were chilled to 10°C during harvest. The harvest volume was 186 L, and the final harvest weight was approximately 2.1 kg of cell paste. An equal amount (w/w) of 50 mM Tris (pH 7.5) and 10% sucrose solution was added to the cell paste. Cells were frozen by pouring the cells suspension into liquid nitrogen, and stored at -20°C, until processed. Quality control results showed 10 out of 10 positive colonies on ampicillin-containing medium in the inoculum, 10/10 positive colonies at induction and 7/10 positive colonies at harvest.

- 115 -

Purification of *T. thermophilus* *hoIB* Product ( $\delta'$ -subunit) Fused to an N-Terminal Peptide That Contains Hexahistidine and a Biotinylation Site by pA1-NB-TD□/MGC1030

5           Lysis was accomplished by creation of spheroplasts of the cells carrying the expressed *T. thermophilus*  $\delta'$ -subunits as described in Example 2. First, from 800 g of a 1:1 suspension of frozen cells (400 g cells) in Tris-sucrose which had been stored at  $-20^{\circ}\text{C}$ , Fr I was prepared (1200 ml, 17 mg/ml). To Fr I, ammonium sulfate (0.194 g to each initial ml Fraction I-35%  
10           saturation) was added over a 15 min interval. The mixture stirred for an additional 30 min at  $4^{\circ}\text{C}$  and the precipitate was collected by centrifugation (23,000 x g, 45 min,  $0^{\circ}\text{C}$ ). The resulting pellets were quick frozen by immersion in liquid nitrogen and stored at  $-80^{\circ}\text{C}$ .

          The pellets from Fr I were resuspended in 150 ml of  $\text{Ni}^{++}$ -NTA  
15           suspension buffer and homogenized using a Dounce homogenizer. The sample was clarified by centrifugation (16,000 x g) and the supernatant constituted Fr II (4 mg/ml). Fr II was added to 50 ml of a 50% slurry of Ni-NTA resin and rocked for 1.5 hours at  $4^{\circ}\text{C}$ . This slurry was then loaded onto a BioRad Econo-column (2.5 x 5 cm). The column was washed with 400 ml of  
20            $\text{Ni}^{++}$ -NTA wash buffer at a flow rate of 1.5 ml/min. The NB-TD' protein was eluted in 200 ml of  $\text{Ni}^{++}$ -NTA elution buffer containing a 10-200 mM imidazole gradient. The eluate was collected in 92 x 2 ml fractions. Fractions were analyzed by SDS-polyacrylamide gel electrophoresis, and fractions 40-75 were found to contain  $\delta'$  that was over 95% pure (FIGs. 27A and 27B).

25           Fractions 40-75 were pooled (70 ml, 0.7 mg/ml) and dialyzed against 2.5 L of HG.04 buffer (20 mM Hepes, (pH 7.6), 40 mM KCl, 1 mM  $\text{MgCl}_2$ , 0.1 mM EDTA, 6 mM  $\beta\text{ME}$ , 10% glycerol). The dialyzed sample constituted Fr III (70 ml, 0.5 mg/ml). From the pool, 75% of the sample was aliquoted, fast frozen in liquid nitrogen and stored at  $-80^{\circ}\text{C}$ . The remaining 25% was  
30           further purified for antibody production.



- 116 -

Production of polyclonal antibodies against *T. thermophilus*  $\delta'$ 

The 25% of *T. thermophilus*  $\delta'$  FrIII discussed above was precipitate by adding ammonium sulfate to 40% saturation (0.226 g of ammonium sulfate per initial ml). The protein pellet was resuspended in 2 ml of 20 mM potassium phosphate, pH 6.5, 100 mM KCl, 25% glycerol and 5 mM DTT buffer. The sample was loaded onto a Sephacryl S-300 column (88 ml, 40:1 height:width ratio) equilibrated in the same buffer. This was accomplished by running the column head down to the resin bed, adding the sample (2 ml), running the sample into the resin and rebuilding the column head. The sample was then eluted in the same buffer at a flow rate of 0.2 ml/min and collected in 1.5 ml fractions. Protein concentrations of each fraction was determined using the Coomassie Protein Assay Reagent (FIG. 28). Fractions were analyzed by SDS-polyacrylamide gel electrophoresis and the fractions were observed to contain homologous  $\delta''$  (FIG. 29).

Fractions 30 to 40 were pooled and the protein was precipitate by adding ammonium sulfate to 40% saturation as previously described. The protein was then resuspended in 3 ml of PBS and dialyzed against 500 ml PBS two times. This constituted Fr IV and was used for antibody production (0.133 mg/ml). The dialyzed samples were used to produce polyclonal antibodies against *T. thermophilus* *holB* gene product ( $\delta'$ -subunit) as described in Example 3.

Construction of Plasmid (pA1-TD') that Overexpresses *T. thermophilus* *holB* gene ( $\delta'$ -Subunit) as a Native Protein

Prior to construction of vector pA1-NB-TD' to express  $\delta'$ -subunit as an N-terminal tagged protein, several attempts were made to first express  $\delta'$  as a native and a C-terminal tagged protein. These attempts were unsuccessful in producing adequate yields of  $\delta'$  to justify purification attempts. These attempts are described in this section.

- 117 -

The *holB* gene was amplified by PCR using pT-TD'-2 as a template and expressed as a native protein. The forward/sense primer (ATG primer #P139-S253, 5'-CTTTCCCCCATGGCTCTACACCCG-3') (SEQ ID NO:44) contained a region complementary to the 5' end of the gene. An *NcoI* site overlapped the ATG start codon. The reverse/antisense primer (ATG primer #P139-A1085, 5'-GGATCCGGCCGGCCTCATCATGTCTCTAAGTCTAAGGC-3') (SEQ ID NO:45) contained an additional stop codon adjacent to the native stop codon, giving two stop codons in tandem. There is a *FseI* site adjacent to the second stop codon and a *BamHI* restriction site adjacent to the *FseI* restriction site. This PCR fragment was digested with *NcoI* and *FseI* restriction enzymes and inserted into the plasmid pA1-CB-*NcoI* digested with the same two enzymes. The plasmid was resealed and transformed into DH5 $\alpha$  bacteria. Plasmids from ampicillin-resistant positive isolates were screened by *NcoI* and *FseI* restriction digest yielding 0.8 and 5.6 kb fragments. The sequence of the inserted region was confirmed by DNA sequencing (ATG SEQ #1447-1450; primers, P38-S5576, P65- A106, P139-S651 and P139-A681). The sequence of the clone showed unexpected extra bases downstream of the *FseI* restriction site, although the remainder of the insert had the correct sequence. Therefore, the *NcoI/FseI* fragment contained the correct sequence. This plasmid was named pA1-TD'(a) and the isolate was stored as a stock culture (ATG glycerol stock #844). To insure that the downstream region contained the correct sequence, pA1-TD'(a) was digested with *NcoI/FseI* restriction enzymes and inserted into another pA1-CB-*NcoI* plasmid digested with the same restriction enzymes. This plasmid was resealed and also transformed into DH5 $\alpha$  bacteria. Plasmids from ampicillin-resistant colonies were again screened by *NcoI* and *FseI* restriction digest yielding 0.8 and 5.6 kb fragments. The sequence of the inserted region was again confirmed by DNA sequencing (ATG SEQ #1473-1476, 1485; primers, P38-S5576, P65- A106, P139-S651, P139-A681 and P139-S321). Sequence analysis confirmed the correct

- 118 -

sequence throughout the region sequenced. This plasmid was named pA1-TD' and the isolate was stored as a stock culture (ATG glycerol stock #878).

5 Verification of Expression of Plasmid (pA1-TD') that Overexpresses *T. thermophilus* *holB* gene ( $\delta'$ -Subunit) as a Native Protein from pA1-TD'/MGC1030

10 Plasmid pA1-TD' was prepared from DH5 $\alpha$  bacteria. The plasmid was transformed into MGC1030 bacteria (ATG glycerol stock #893, 894, 895). The bacterial growths of three isolates and isolation of total cellular protein were as described in Example 2. A small aliquot (3  $\mu$ l) of supernatant containing total cellular protein from each of the three isolates was electrophoresised onto a 4-20% SDS-polyacrylamide mini-gel (Novex, EC60255; 1 mm thick, with 15 wells/gel) in 25 mM in Tris base, 192 mM  
15 glycine, and 0.1% SDS. The mini-gel was stained with Coomassie Blue. There were no visible protein bands from any of the isolates corresponding to the predicted molecular weight of *T. thermophilus*  $\delta'$ .

20 Construction of a Plasmid (pA1-CB-TD') that Overexpresses *T. thermophilus* *holB* ( $\delta'$ -subunit) Fused to a C-Terminal Peptide That Contains Hexahistidine and a Biotinylation Site

Again, since attempts to express native  $\delta'$  failed, expression of this protein was attempted by coupling the protein to a C-terminal fusion peptide.  
25 The gene encoding *T. thermophilus* *holB* above was amplified by PCR using pT-TD'-2 plasmid as a template. The forward/sense primer (ATG primer #P139-S253) was the same primer used in construction of pA1-TD' and contained a region complementary to the 5' end of the *T. thermophilus* *holB* gene. As before, an *Nco*I site overlapped the ATG start codon. The  
30 reverse/antisense primer was complementary to the 3' end of the *T. thermophilus* *holB* gene excluding the stop codon (ATG primer #P139-A1075, 5'-GAGGACTAGTTGTCTCTAAGTCTAA GGC -3') (SEQ ID NO:46). This primer contained a *Spe*I restriction site adjacent to the complementary

- 119 -

region of the primer. The *SpeI* site allowed for the expressed protein to contain two additional amino acids (Thr and Ser) between the C-terminal amino acid of the  $\delta'$ -subunit and the C-terminal fusion peptide. This 822 bp PCR product was digested with *NcoI* and *SpeI* and inserted into the plasmid pA1-CB-*NcoI* digested with the same restriction enzymes. This plasmid was transformed into DH5 $\alpha$  bacteria and plasmids from ampicillin-resistant positive isolates were screened for by digestion with *NcoI* and *SpeI* restriction enzymes yielding 0.8 and 5.6 kb fragments. The sequence of the insert was verified by DNA sequencing (ATG SEQ #1500-1503; primers, P38-S5576, P65-A106, P139-S651, P139-S321). This plasmid was named pA1-CB-TD $\square$  and the isolate was stored as a stock culture (ATG glycerol stock #896).

Verification of Expression of *T. thermophilus*  $\delta'$ -subunit Fused to a C-Terminal Peptide That Contains Hexahistidine and a Biotinylation Site by pA1-CB-TD'/MGC1030

The pA1-CB-TD' plasmid was prepared and transformed into MGC1030 bacteria (ATG glycerol stock #920). The bacterial growths of three isolates and isolation of total cellular protein were as described in Example 2. A small aliquot of each supernatant (3  $\mu$ l) containing total cellular protein was electrophoresised onto a 4-20% SDS-polyacrylamide mini-gel (Novex, EC60255; 1 mm thick, with 15 wells/gel) in 25 mM Tris base, 192 mM glycine, and 0.1% SDS. The mini-gels were stained with Coomassie Blue. The region of the gel in which  $\delta'$  was expected contained other intense protein bands and  $\delta'$  could not be visualized.

Next, the total protein in each lysate was transferred (blotted) from polyacrylamide gel to nitrocellulose as described in Example 2. Each lane contained 1.5  $\mu$ l of the supernatant. Proteins on the blotted nitrocellulose were visualized by interactions with phosphatase-conjugated streptavidin as described above. The endogenous *E. coli* biotin-CCP, ~20 kDa, was detectable in both induced and non-induced samples. A very faint protein band corresponding to  $\delta'$  migrated midway between the 30 and 40 kDa

- 120 -

molecular weight standard of the Gibco 10 kDa protein ladder. The predicted molecular weight of  $\delta'$  is 33 kDa. This protein was observed as a faint band in the induced cultures, but was not observed in the uninduced control in lysates. *T. thermophilus*  $\delta'$  was not expressed in great enough quantities to justify growth and purification.

Cloning *T. thermophilus* *holB* gene ( $\delta'$ ) into a translationally coupled vector pTAC-CCA-*ClaI*

To efficiently express  $\delta'$  as a native protein we designed a vector to express  $\delta'$  as a translationally coupled protein. The goal is to use translational coupling as described in Example #2. The *holB* gene was inserted behind the CCA adding enzyme and translationally coupled in two steps. First, the *holB* gene was amplified by using pA1-TD' as a template by PCR. The forward/sense primer (ATG primer #P139-S250cla2, 5'-ACTGATCGATAATGGCTCTACACCCGGCTCACCC-3') (SEQ ID NO:57) has a *ClaI* restriction site in the non-complementary region. The non-complementary region also contains the "TA" of the stop (TAA) for the upstream CCA-adding protein fragment. The region of the primer complementary to the 5' end of the *T. thermophilus* *holB* gene begins with "A" which is the first nucleotide of the "ATG" start codon and the final "A" of the "TAA" stop codon. The reverse/antisense primer (ATG primer #P139-A1081stopspe, 5'-GGACACTAGTTCATCATGTCTCTAAGTCTAA-3') (SEQ ID NO:58) contains a *SpeI* restriction site in the non-complementary portion of the primer and also an additional stop codon adjacent to the native stop codon, giving two stop codons in tandem. There was also a clamp region for efficient cutting with *SpeI*. In the second step the PCR product was digested with *ClaI/SpeI* restriction enzymes and inserted into the pTAC-CCA-*ClaI* plasmid digested with the same enzymes. The plasmid was transformed into DH5 $\alpha$  bacteria and plasmids from ampicillin-resistant positive isolates were screened for by digestion with *ClaI/SpeI* restriction enzymes yielding 0.8 and 5.5 kb fragments. The sequence of both strands of the insert were verified

- 121 -

by DNA sequencing (ATG SEQ #1737-1742; primers, P144-S23, P65-A106, P139-S321, P139-S651, P139-A681, P139-A1081stopspe). Sequence analysis confirmed that the correct sequence was contained within the inserted region. This plasmid was named pTAC-CCA-TD' and the isolate was stored as a stock culture (ATG glycerol stock #1055).

Verification of expression of native *T. thermophilus*  $\delta'$ -subunit expressed from pTAC-CCA-TD'/MGC1030 and pTAC-CCA-TD'/AP1.L1

The pTAC-CCA-TD' plasmid was prepared and transformed into MGC1030 bacteria (ATG glycerol stock #1083) and AP1.L1 (ATG glycerol stock #1080, 1081, 1082). The bacterial growths and isolation of total cellular protein were as described in Example #2. A small aliquot of each supernatant (3  $\mu$ l) containing total cellular protein was electrophoresised onto a 4-20% SDS-polyacrylamide mini-gel (Novex, EC60255; 1 mm thick, with 15 wells/gel) in 25 mM Tris base, 192 mM glycine, and 0.1% SDS. The mini-gels were stained with Coomassie Blue. The expected location of the *T. thermophilus*  $\delta'$  protein band was in an area containing many bands of native *E. coli* proteins and *T. thermophilus*  $\delta'$  could not be resolved from these other protein bands.

Large Scale Growth of pA1-CCA-TD'/AP1.L1

Strain pA1-CCA-TD'/AP1.L1 was grown in a 250 L fermentor to produce cells for purification of *T. thermophilus*  $\delta'$  as described in Example #2. Optimum induction times were determined as described in Example #2. Cell harvest was initiated 3 hours after induction, at OD<sub>600</sub> of 3.12, and the cells were chilled to 10°C during harvest. The harvest volume was 175 L, and the final harvest weight was approximately 1.37 kg of cell paste. An equal amount (w/w) of 50 mM Tris (pH 7.5) and 10% sucrose solution was added to the cell paste. Quality control results showed 10 out of 10 positive colonies on ampicillin-containing medium in the inoculum and 10/10 positive colonies at

- 122 -

induction and 10/10 positive colonies at harvest. Cells were frozen by pouring the cells suspension into liquid nitrogen, and stored at -20°C, until processed.

#### Purification of native *T. thermophilus* $\delta'$ from pA1-CCA-TD'

5

Lysis was accomplished by creation of spheroplasts of the cells carrying the expressed *T. thermophilus*  $\delta'$ . First, from 400 g of a 1:1 suspension of frozen cells (200 g cells) in Tris-sucrose which had been stored at -20 °C, FrI was prepared (700 ml, 13.7 mg/ml). The preparation was as described in Example #2. To Fr I, ammonium sulfate (0.258 g to each initial ml Fraction I-45% saturation) was added over a 15 min interval. The mixture stirred for an additional 30 min at 4 °C and the precipitate was collected by centrifugation (23,000 x g, 45 min, 0 °C). The resulting pellets were quick frozen by immersion in liquid nitrogen and stored at -80 °C.

10

15

In the following purification steps, fractions from purification columns were assayed using the reconstitution assay (described in Example 7) to determine fractions that contained activity and therefore the  $\delta'$  -subunit. One-half of the pellets from Fr I was resuspended in 270 ml of 50 mM Tris-HCl, (pH 7.5), 25% glycerol, 1 mM EDTA, 1 mM DTT and homogenized using a Dounce homogenizer. The sample was clarified by centrifugation (16,000 x g) and the supernatant constituted Fr II (270 ml, 7.7 mg/ml). Fr II was further purified using a Butyl Sepharose Fast Flow (Pharmacia Biotech) column. The butyl resin (400 ml) was equilibrated in butyl equilibration buffer (50 mM Tris-HCl, (pH 7.5), 25% glycerol, 1 mM EDTA, 1 mM DTT, 0.5 M ammonium sulfate). The column was poured using 260 ml of butyl resin. The remaining 140 ml of butyl resin was mixed with Fr II giving 410 ml. To this mixture, saturated ammonium sulfate (0.5 sample volume) was added slowly while stirring over a 1 hour period. This mixture was added to the column at 1.3 ml/min. The column was then washed with 4 L of butyl equilibration buffer. The protein was eluted in 10 column volumes of a gradient beginning with butyl equilibration buffer and ending in a buffer containing 50 mM Tris-

20

25

30

- 123 -

HCl, (pH 7.5), 25 % glycerol, 1 mM EDTA, 1 mM DTT, 50 mM KCl. Remaining protein was removed from the column by eluting with a additional 7.5 column volumes "bump" of the end buffer. The  $\delta'$ -subunit eluted in the first half of the "bump", and was pooled (485 ml, 0.1 mg/ml). The other one-half of the pellets from Fr I was purified exactly the same as describe. The two preparations were combined to give 972 ml (0.1 mg/ml) of Fr III.

Fr III was further purified using an Octyl Sepharose Fast Flow (Pharmacia Biotech) column. The octyl resin (20 ml) was equilibrated in octyl equilibration buffer (50 mM Tris-HCl, (pH 7.5), 10% glycerol, 1 mM DTT, 1 mM EDTA, 0.5 M ammonium sulfate). The column was poured using 13 ml of octyl resin. The remaining 7 ml of octyl resin was mixed with Fr III giving 979 ml. To this mixture saturated ammonium sulfate (0.5 sample volume) was added slowly while stirring over a 1 hour period. This mixture was added to the column at 1.3 ml/min. The column was then washed with 600 ml of octyl wash buffer (50 mM Tris-HCl, (pH 7.5), 10% glycerol, 1 mM DTT, 1 mM EDTA, 200 mM ammonium sulfate). The wash was collected in fractions (10 ml). The protein was eluted in 10 column volumes (200 ml) of a gradient beginning with octyl wash buffer and ending in a buffer containing 50 mM Tris-HCl, (pH 7.5), 25 % glycerol, 1 mM EDTA, 1 mM DTT, 50 mM KCl. The  $\delta'$ -subunit was recovered in fractions making up the wash. These fractions were pooled (210 ml, 0.07 mg/ml) and concentrated using PEG 8000 and constitute Fr IV (38 ml, 0.26 mg/ml).

*T. thermophilus*  $\delta'$  was further purified using a Sephacryl S300 HR (Pharmacia Biotech) gel filtration column (510 ml, 3 cm x 120 cm) equilibrated in 50 mM Tris-HCl, (pH 7.5), 20 % glycerol, 100 mM NaCl, 1 mM EDTA, 5 mM DTT. The column was loaded and the protein eluted at a flow rate of 0.7 ml/min. The  $\delta'$  -subunit was isolated as a highly purified protein (54 ml, 0.08 mg/ml). The products of the different purification steps for  $\delta'$  expressed as a translationally coupled protein were analyzed by a SDS-polyacrylamide gel (FIG. 30).



- 124 -

## EXAMPLE 6

Construction of a Plasmid (pA1-NB-TN) that Overexpresses *T. thermophilus*  
*dnaN* ( $\beta$ -subunit) Fused to an N-Terminal Peptide That Contains  
Hexahistidine and a Biotinylation Site

5

In *E. coli* the  $\beta$ -subunit is functional as a homodimer (see Johanson, K.O. and Charles S. "Purification and Characterization of the  $\beta$ -subunit of the DNA Polymerase III Holoenzyme of *Escherichia coli*", *J. Biol. Chem.*, 255:10984-10990 (1980)). This dimer confers the ability of high processive  
10 synthesis to the core polymerase. In the previous patent application (U.S. Application # 09/151888), the identification of the *T. thermophilus* gene (*dnaN*) encoding the  $\beta$ -subunit was described. From the lambda vector preparation described in Example 9 of the previous application (U.S. Application # 09/151888), the 2.2 kb *DraI/EcoRI* fragment was cloned into a  
15 pBluescript II SK<sup>+</sup> vector (Stratagene). This sub-clone was then transformed into XL I Blue cells. A total of ten ligation reactions were attempted before achieving a successful clone. This clone (UCO9) was grown and plasmid DNA was isolated. Both strands of the DNA from the inserted region was sequenced (Lark Technologies Inc., DNA SEQ# UC0: 9.2.T7X, 9.23.AP124,  
20 9.AP110, 9.2.AP114, 9.23.AP125, 9.2.AP112, 9.2.AP113, 9.23.AP128, 9.6.AP119, 9.25.AP35, 9.6.AP118, 9.25.AP36, 9.23.AP126, 9.25.AP34B, 9.23.AP32B, 9.6.AP121, 9.25.AP40, 9.23.AP121, 9.2.-48R, 9.6.AP116, 9.23.AP131, 9.6.AP122, 9.23.AP122). Each nucleotide in the inserted region was confirmed using at least three individual primers. This sub-clone was  
25 designated UCO9.

In the DNA coding sequence of the *T. thermophilus dnaN* gene (SEQ ID NO:22) (FIG. 62) the start codon (atg) and the stop codon (tag) are in bold print. The 5' and 3' UTRs are also shown (lower case). Also shown is the protein (amino acid) sequence (SEQ ID NO:23) (FIG. 63) derived from the  
30 DNA coding sequence.

To simplify purification, the  $\beta$ -subunit coupled to an N-terminal fusion peptide that contains hexahistidine and a biotinylation site was expressed first.

- 125 -

Plasmids were designed to fuse the *dnaN* gene to DNA encoding an N-terminal peptide that contains hexahistidine and a biotinylation. First, a PCR fragment containing the 5'-portion of the *Tth dnaN* gene was amplified from plasmid UCO9 using a forward primer (ATG #P118-S85, 5'-  
5 AACTGCAGAACATAACGGTTCCTCAAGAACTCC-3') (SEQ ID NO:24) that adds a *Pst*I site to the 5'-end of the gene so that the actual PCR product excluded the ATG start codon and begins at codon 2. The underlined region of forward primers indicates nucleotides that are complementary to the 5' end of the gene, here and in all other primers used. The *Pst*I site is adjacent to  
10 codon 2, so that when this fragment was inserted into the pAI-NB Age-1 plasmid the *dnaN* gene was in frame with the DNA encoding the N-terminal fusion peptide. The reverse primer (ATG #P118-A731, 5'-  
GACCCGCACCATCTCGTCCACG-3') (SEQ ID NO:25) is downstream of the *Sac*II restriction site (which is near position 496 downstream of the ATG start codon). The resulting PCR product was digested with *Pst*I and *Sac*II and  
15 ligated into the *Pst*I/*Sac*II cut pAI-NB Age-1 and transformed into DH5 $\alpha$ . Plasmids from ampicillin-selected positive isolates were verified by digestion with *Pst*I/*Sac*II restriction digestion yielding the expected 0.5 and 5.5 kb fragments. This plasmid (pAI-NB-TN5') was sequenced across the PCR  
20 inserted regions to confirm the correct sequence (ATG SEQ #1187-1190, primers P64-S10, P64-A215, P118-S290 and P118-A411). This sequence was also compared to that from the UCO9 insert. This precursor plasmid was named pAI-NB-TN5' and the positive isolate (pAI-NB-TN5'/ DH5 $\alpha$ ) was stored as a stock culture (ATG glycerol stock #708).

25 The 3' region (C-terminus) of the *T. thermophilus dnaN* gene was cut out of the UCO9 plasmid in a partial digest using the two restriction enzymes *Sac*II and *Nco*I. The *Nco*I digested site is approximately 150 bases downstream of the stop codon. This gave a fragment size of approximately 800 bases. There is also a second *Sac*II restriction site further downstream of  
30 the *Nco*I restriction site approximately 400 bases. This second site gave an additional fragment of approximately 400 bases in length. The proper

- 126 -

fragment was easily identified, as it was twice as large as the fragment given by the secondary *SacII* restriction site. The fragments were separated by electrophoresis, and the 800 bp fragment was eluted in water. This fragment containing the 3' portion of the TN gene was inserted into the pA1-NB-TN5' plasmid that had been digested with both *SacII* and *NcoI* restriction enzymes. This plasmid (pAI-NB-TN) contained the entire *T. thermophilus dnaN* fused to the DNA encoding an N-terminal fusion peptide. This plasmid was transformed into DH5 $\alpha$ . Plasmids from ampicillin-resistant colonies were verified by cleavage with *SacII/NcoI* yielding the expected 6.1 kb and 0.8 kb fragments. The positive isolate (pAI-NB-TN/ DH5 $\alpha$ ) was stored as a stock culture (ATG glycerol stock #722).

#### Verification of Expression of *T. thermophilus* $\beta$ -subunit Fused to an N-Terminal Peptide That Contains Hexahistidine and a Biotinylation Site

pA1-NB-TN was prepared and transformed into MGC1030 (ATG glycerol stock #765) and AP1.L1 bacteria (ATG glycerol stock #743). The bacterial growths of three isolates and isolation of total cellular protein were as described in Example 2. An aliquot (4  $\mu$ l) of each supernatant containing total cellular protein was loaded onto a 4-20% SDS-polyacrylamide mini-gel (Novex, EC60255; 1 mm thick, with 15 wells/gel) in 25 mM in Tris base, 192 mM glycine, and 0.1% SDS. The mini-gels were stained with Coomassie Blue. The region of the gel in which N-tagged *T. thermophilus*  $\beta$  was expected contained many other very intensely stained protein bands and N-terminal tagged *T. thermophilus*  $\beta$  could not be visualized.

The total protein in each lysate was analyzed by biotin blot analysis as described in Example 2. The endogenous *E. coli* biotin-CCP, ~20 kDa was detectable in both induced and non-induced samples. A protein band corresponding to the  $\beta$ -subunit migrated approximately midway between the 40 and 50 molecular weight standards of the Gibco 10 kDa protein ladder. This protein was observed as a distinct band in the induced cultures, but was

- 127 -

not observed in the uninduced control in lysates from the AP1.L1 strain. No expression could be detected in the MGC1030 strain.

#### Optimization of Expression of *T. thermophilus* *dnaN* gene ( $\beta$ -subunit)

5

Expression was analyzed using the bacterial strains AP1.L1 carrying the pA1-NB-TN plasmid at different induction times and also at different growth temperatures (25°C and 37°C). Growth of bacterial cultures and analysis were carried out as described in Example 2. Biotin blot analysis indicated that expression levels were highest at 37°C (FIG. 31). Since SDS-polyacrylamide gel electrophoresis indicates that most of the  $\beta$ -subunit is being expressed in 4 hours and at 37°C, these growth condition were used in subsequent preparations.

10

15

#### Large Scale Growth of pA1-NB-TN/AP1.L1

Strain pA1-NB-TN/AP1.L1 was grown in a 250 L fermentor to produce cells for purification of *T. thermophilus*  $\beta$ -subunit as described in Example 2. Cell harvest was initiated 4 hours after induction, at OD<sub>600</sub> of 6.7, and the cells were chilled to 10°C during harvest. The harvest volume was 180 L, and the final harvest weight was approximately 2.2 kg of cell paste. An equal amount (w/w) of 50 mM Tris (pH 7.5) and 10% sucrose solution was added to the cell paste. Cells were frozen by pouring the cells suspension into liquid nitrogen, and stored at -20°C, until processed. Quality control results showed 10 out of 10 positive colonies on ampicillin-containing medium in the inoculum and 10/10 positive colonies at harvest.

20

25

- 128 -

### Determination of Optimal Ammonium Sulfate Precipitation Conditions for N-terminal Tagged *T. thermophilus* $\beta$

Lysis was accomplished by creation of spheroplasts of the cells carrying the expressed *T. thermophilus*  $\beta$ -subunits. First, from 100 g of a 1:1 suspension of frozen cells (50 g cells) in Tris-sucrose which had been stored at  $-20^{\circ}\text{C}$ , FrI was prepared (390 ml, 9.8 mg/ml). The preparation was as described in Example 2. FrI was divided into 5 equal volumes and 0.164, 0.226, 0.291, 0.361 and 0.436 g of ammonium sulfate (30%, 40%, 50%, 60% and 70% saturation) was added to each separate sample, respectively, over a 15 min interval at  $4^{\circ}\text{C}$ . The mixture stirred for an additional 30 min at  $4^{\circ}\text{C}$  and the precipitate was collected by centrifugation ( $23,000 \times g$ , 45 min,  $0^{\circ}\text{C}$ ). The resulting pellets were resuspended in 1 ml Ni-NTA suspension buffer (50 mM Tris-HCl (pH 7.5), 40 mM KCl, 7 mM  $\text{MgCl}_2$  and 10% glycerol). The protein concentration of each sample was determined using the Coomassie Protein Assay Reagent (Pierce) and bovine serum albumin (BSA) as a standard. The 30%, 40%, 50%, 60% and 70% ammonium sulfate precipitated samples contained protein concentrations of 2.4, 8.0, 18.0, 35.0 and 38.0 mg/ml, respectively. The samples were analyzed by SDS-polyacrylamide gel electrophoresis. The 40% ammonium sulfate precipitated samples contained over 90% of the  $\beta$ -subunit, this concentration of ammonium sulfate was used in subsequent preparations.

### Purification of *T. thermophilus* N-Terminal Tagged $\beta$

Lysis was accomplished by creation of spheroplasts of the cells carrying the expressed *T. thermophilus*  $\beta$ -subunits. First, from 600 g of a 1:1 suspension of frozen cells (300 g cells) in Tris-sucrose which had been stored at  $-20^{\circ}\text{C}$ , FrI was prepared (1.05 L, 15.4 mg/ml). The preparation was as described in Example 2. To Fr I, ammonium sulfate (0.266 g to each initial ml Fraction I-40% saturation) was added over a 15 min interval. The mixture was stirred for an additional 30 min at  $4^{\circ}\text{C}$  and the precipitate was collected by

- 129 -

centrifugation (23,000 x g, 45 min, 0°C). The resulting pellets were quick frozen by immersion in liquid nitrogen and stored at -80°C.

The pellets from FrI ammonium sulfate precipitation were resuspended in 100 ml of Ni<sup>++</sup>-NTA suspension buffer and homogenized using a Dounce homogenizer. The sample was clarified by centrifugation (16,000 x g) and the supernatant constituted Fr II (19.5 mg/ml, 100 ml). Fr II was added to 30 ml of a 50% slurry of Ni-NTA resin and rocked for 1.5 hours at 4°C. This slurry was then loaded onto a BioRad Econo-column (2.5 x 5 cm). The column was washed with 200 ml of Ni<sup>++</sup>-NTA wash buffer at a flow rate of 0.5 ml/min. The N-terminal tagged  $\beta$  was eluted with a 150 ml 10-200 mM imidazole gradient in Ni<sup>++</sup>-NTA elution buffer. The eluate was collected in 75 x 2 ml fractions. Fractions were analyzed by SDS-polyacrylamide gel electrophoresis, and fractions 26-60 were found to contain over 90% of total  $\beta$ -subunit protein (FIG. 32). These fractions also contained most of the ability to stimulate the  $\beta$ -subunit in primer extension assays (discussed below).

Fractions 26-60 were pooled (67 ml) and dialyzed two times against 1 L of buffer HG.04 (20 mM Hepes (pH 7.0), 40 mM KCl, 1 mM MgCl<sub>2</sub>, 0.1 mM EDTA, 10% glycerol and 6 mM  $\beta$ ME). The sample constituted FrIII (65 ml, 3.8 mg/ml), which was aliquoted and fast frozen in liquid nitrogen and stored at -80°C.

#### Development of a Simple Processivity Assay for *T. thermophilus* $\beta$ on a Defined Linear Template

Replicative polymerases ranging from *E. coli* to yeast are stimulated by their cognate "sliding clamp processivity factors",  $\beta$  and PCNA respectively, in the absence of other holoenzyme subunits if they are present at high non-physiological concentrations on linear templates (see Crute, J.J., *et al.*, *J.Biol.Chem.* 258:11344-11349 (1983)). This is due to the ability to these factors to assemble on linear DNA in the absence of the clamp loader (DnaX or RFC) at high concentrations. To develop an assay for detection of *T.*

- 130 -

*thermophilus*  $\beta$  we have taken advantage of the low processivity of DNA replicative polymerases in the absence of other members of the replicative complex. In the absence of  $\beta$  the DNA polymerase ( $\alpha$ -subunit) will only extend a primer by approximately 10 nucleotides per each binding event (see Crute, J.J., *et al.*, *J.Biol.Chem.* 258:11344-11349 (1983)). The substrate (shown below) (SEQ ID NO:28) allows detection of stimulation by  $\beta$ .

5' -TGCAAATCGCGTTAGCTTAG-3'

(EO-8)

3' -ACGTTTAGCGCAATCGAATCTGTCCTGTGTGTTCCCTGCTGCTCCGTTTCAAAAAAAAAA

AAAAAAAAAAAA-5'

(EO-7)

*T. thermophilus*  $\beta$  would be expected to bind the annealed primer/template and extend the primer for a relatively short distance per binding event in the absence of  $\beta$ . The template lacks "A"s for the first 30 nucleotides and then contains a string of "A"s. If replication is allowed to proceed in a large excess of template primer and limiting polymerase, a template, on average, will only encounter a polymerase once during the course of the assay. Thus, in the absence of  $\beta$  *T. thermophilus*  $\alpha$  would not be expected to incorporate significant levels of radiolabeled dTTPs opposite the terminal sequence of "A"s. Therefore, it should be possible to use this system to detect stimulation of the processivity of the DNA polymerase in the presence of  $\beta$ .

To allow annealing, the template (EO7) and primer (EO8) were diluted to 10  $\mu$ M each in annealing buffer (10 mM Tris-HCl, pH 7.5, 0.1 mM EDTA), heated to 90°C in a heating block and allowed to slowly cool to room temperature. Reactions (25  $\mu$ l) were carried out at 30°C for 5 min in enzyme dilution buffer (EDB) (50 mM Hepes (pH 7.5), 20% glycerol, 0.02% Nonidet P40, 0.2 mg/ml BSA, 10 mM DTT, 10 mM  $MgCl_2$ ), dNTP mix (50  $\mu$ M dATP, dCTP, dGTP and 18  $\mu$ M [ $^3H$ ]dTTP, 100 cpm/pmol) and varying amounts of DNA polymerase (1  $\mu$ l),  $\beta$  and annealed DNA.

- 131 -

In reactions using *E. coli* DNA polymerase  $\alpha$ , the concentration of primer/template was varied between 0.1-1.3  $\mu$ M to determine the amount needed to maintain the level of incorporation of radioactivity to that of the background signal, due to single binding events. These reactions were carried out in the absence of  $\beta$  at 0.3, 0.6, and 1.2 nM  $\alpha$ . There was no increase in the total dTTP incorporated between 0.6 and 1.3  $\mu$ M of primer/template. Therefore, in reactions to optimize levels of *T. thermophilus*  $\alpha$ , 1.3  $\mu$ M primer/template was used.

To determine the optimum amount of *T. thermophilus* polymerase (1 mg/ml) to use, assays were set up using 100, 250, 500, 1000, 2000 and 4000:1 dilution ratios of *T. thermophilus* N-terminal tagged *T. thermophilus*  $\alpha$  (1-4  $\mu$ l polymerase/reaction). The samples containing 250:1 dilution of *T. thermophilus*  $\alpha$  gave a signal equal to the background signal, therefore this concentration of N-terminal tagged *T. thermophilus*  $\alpha$  was used in reactions to screen for  $\beta$  stimulation.

To assay for the ability of various amounts of N-terminal tagged *T. thermophilus*  $\beta$  to stimulate the activity of the *T. thermophilus*  $\alpha$ , the primer-extension assay was used. Using a 250:1 dilution of the  $\alpha$ -subunit (1 mg/ml) and 1.3  $\mu$ M of annealed primer/template, assays were carried out at 0, 0.25, 0.5, 1.0, 2.0 and 4.0  $\mu$ M *T. thermophilus*  $\beta$  (FIG. 33). *T. thermophilus*  $\alpha$  was stimulated by increasing concentrations of  $\beta$  (FIG. 33) consistent with a functional  $\beta$ , proving the capability of purified *T. thermophilus*  $\alpha$  and  $\beta$  to cooperate in a processive replicative reaction at elevated temperatures.

#### Production of polyclonal antibodies against *T. thermophilus* $\beta$

From N-terminal tagged *T. thermophilus*  $\beta$  FrIII described in the section entitled "Purification of *T. thermophilus* *dnaN* Product ( $\beta$ -subunit) Fused to an N-Terminal Peptide That Contains Hexahistidine and a Biotinylation Site by pA1-NB-TN/AP1.L1", 2 ml (3.8 mg/ml) was loaded



- 132 -

onto an Sephacryl S-300 column (88 ml, 40:1 height:width ratio) equilibrated in 20 mM potassium phosphate, pH 6.5, 100 mM KCl, 25% glycerol and 5 mM DTT. This was accomplished by running the buffer above the column bed down to the resin bed, adding the sample (2 ml), running the sample into the resin and rebuilding the buffer above column bed. The sample was then eluted in the same buffer at a flow rate of 0.2 ml/min and collected in 1 ml fractions. Protein concentrations of each fraction was determined using the Coomassie Protein Assay Reagent (FIG. 34). The fractions were analyzed by SDS-polyacrylamide gel electrophoresis and fractions 43-66 were pooled (20 ml, 0.15 mg/ml) (FIG. 35).

Protein in the pooled fractions were precipitated by addition of ammonium sulfate (0.436 g to each ml of pooled fractions-70% saturation) and the precipitate was collected by centrifugation (23,000 x g, 45 min, 0°C). The ammonium sulfate precipitated pellets were dissolved in 2 ml of PBS (0.24 mg/ml) and dialyzed against 500 ml of PBS two times. This dialyzed sample was analyzed by SDS-polyacrylamide gel electrophoresis (FIG. 36).

Polyclonal antibodies against *T. thermophilus*  $\beta$  were produced by inoculation of a rabbit with N-terminal tagged *T. thermophilus*  $\beta$  and harvested from the rabbit as described in Example 3. The optimum dilution of anti-serum for binding N-tagged *T. thermophilus*  $\beta$  was determined after the test bleed and after the final bleed. This was carried out by SDS-polyacrylamide gel electrophoresis, in which a small aliquot of N-terminal tagged *T. thermophilus*  $\beta$  (0.5  $\mu$ g/well) was electrophoresed onto a 10% SDS-polyacrylamide mini-gel (10 x 10 cm). The protein was transferred onto nitrocellulose membrane as described above in Example 3. The membrane was cut into strips with each strip containing an identical band of N-terminal tagged *T. thermophilus*  $\beta$ . The membrane was blocked in 0.2% Tween 20 (v/v)-TBS (TBST) containing 5% non-fat dry milk (w/v) for 1 hour at room temperature, rinsed with TBST. The strips were placed in antiserum/TBST (dilutions: 1:100, 1:200, 1:400, 1:800, 1:1600, 1:3200, 1:6400, and 1:12800) for 1 hour and then washed 4 times for 5 min in TBST. Next, the strips were

- 133 -

placed in secondary antibody-conjugated to alkaline phosphatase (goat anti-rabbit IgG (H+L), 1:3000 dilution in TBST) (BioRad) for 1 hour. The strips were then washed 4 times for 5 min with TBST. Following this extensive washing, the blots were developed with BCIP/NBT (KPL #50-81-07; one component system). Proteins corresponding to  $\beta$  were visualized as distinct bands even at the highest dilution of antiserum (FIG. 37). These bands became more intense as the dilution of antiserum was decreased. The negative control contained antiserum taken from the rabbit prior to inoculating with antigen. The positive control is a biotin blot analysis of the antigen at the same concentration (0.5  $\mu$ g) as used in antiserum detection.

Next, the minimum amount of  $\beta$  needed for recognition by antibody serum was determined. This was carried out using SDS-polyacrylamide gel electrophoresis in which small aliquots of  $\beta$  (0.02, 0.04, 0.08, 0.16, 0.32, 0.64, 1.25, 2.50, and 5.0  $\mu$ g/well) were electrophoresed onto a 10% SDS-polyacrylamide mini-gel (10 x 10 cm). The protein was transferred onto nitrocellulose membrane. The blotted nitrocellulose was blocked in TBST containing 5% non-fat dry milk (w/v) for 1 hour at room temperature, rinsed with TBSt. The blot were placed in antiserum/TBST (dilution of 1:6400) for 1 hour and then washed 4 times for 5 min in TBSt. Next, the blot was placed in secondary antibody-conjugated to alkaline phosphatase (goat anti-rabbit IgG (H+L), 1:3000 dilution in TBST) (BioRad) for 1 hour. The blot was then washed 4 times for 5 min with TBST. Following this extensive washing, the blot was developed with BCIP/NBT (KPL #50-81-07; one component system) (FIG. 38). The lowest level (0.02  $\mu$ g) of N-tagged *T. thermophilus*  $\beta$  could be detected.

#### Construction of a Plasmid (pA1-TN) that Overexpress Native *T. thermophilus* *dnaN* ( $\beta$ -subunit)

To express native (un-tagged) *T. thermophilus*  $\beta$  the *dnaN* gene was inserted into the vector pA1-CB-NdeI. The C-terminal biotin-hexahis tag

- 134 -

carried by this plasmid will be downstream and out of frame with the inserted *dnaN* gene. The forward primer was designed so that CAT was added in the non-complementary region of the primer immediately proceeding the ATG start codon. This resulted in CATATG, an *NdeI* restriction site (ATG primer #P118-S74, 5'-GGATCCAAGCTTCATATGAACATAACGGTTCCCAAG AAA-3') (SEQ ID NO:41). The reverse primer was designed so that an additional stop codon was added in the non-complementary region producing two stop codons in tandem. The non-complementary region of the reverse primer contains an *NheI* restriction site and additional nucleotides for efficient digestion of the PCR product with the restriction enzyme (ATG primer #P118-1231, 5'-GAGCAGCTAGCCTACTAGACCCTGAGGGGCACCAC-3') (SEQ ID NO:42). The PCR reaction resulted in a product which contained the entire *T. thermophilus dnaN* gene with an *NdeI* site overlapping the start codon and an additional stop codon in tandem with the natural stop codon (TAG) and an *NheI* site downstream of the tandem stop. Digestion of the PCR product and the pGEM-T Easy plasmid with *NdeI* and *NheI* allowed the *T. thermophilus dnaN* gene to be inserted the pGEM-T Easy plasmid. The PCR product was ligated into the pGEM-T Easy plasmid as a preliminary plasmid for sequencing of the insert region. This plasmid was transformed into DH5 $\alpha$ , and ampicillin-resistant positive isolates were selected. Plasmids from one positive isolate was isolated and screened by *EcoRI* digestion of plasmids yielding 1.15 and 3.0 kb fragments. The correct sequence of both DNA strands of the insert containing the *dnaN* gene were verified by DNA sequencing (ATG SEQ #1420-1427; primers, SP6 sequencing primer, T7 sequencing primer, P118-S290, P118-S639, P118-S1003, P118-A996, P118-A731 and P118-A411). This sequence was compared to the sequence obtained in the section entitled "Construction of a Plasmid (pA1-NB-TN) that Overexpresses *T. thermophilus dnaN* ( $\beta$ -subunit) Fused to an N-Terminal Peptide That Contains Hexahistidine and a Biotinylation Site". This plasmid was named pT-TN and the positive isolate (pT-TN/ DH5 $\alpha$ ) was stored as a stock culture (ATG glycerol stock #839).

- 135 -

The *T. thermophilus dnaN* gene was recovered from the preliminary pT-TN plasmid and inserted into an expression vector. The pT-TN plasmid was digested with *NdeI/NheI* restriction enzymes and the entire TN gene was inserted into the pA1-CB-*NdeI* plasmid digested with the same restriction enzymes. This placed the *dnaN* gene into the pA1-CB-*NdeI* plasmid out of frame with the downstream biotin-hexahis tag. This also placed the start codon 11 nucleotides downstream of the RBS. The plasmid was transformed into DH5 $\alpha$  and positive isolates were selected by ampicillin-resistance. Plasmid from one positive clone was verified by *NdeI/NheI* and *XbaI* restriction digest yielding the expected 1.1 and 5.6 kDa and 0.1 and 6.7 kDa fragments, respectively. The sequence of the inserted region was confirmed by DNA sequencing (ATG SEQ #1443 and #1444, primers P118-S1003 and P38-S5576). This sequence was compared to the sequence obtained in the section entitled "Construction of a Plasmid (pA1-NB-TN) that Overexpresses *T. thermophilus dnaN* ( $\beta$ -subunit) Fused to an N-Terminal Peptide That Contains Hexahistidine and a Biotinylation Site". This plasmid was named pA1-TN and the isolate (pA1-TN/ DH5 $\alpha$ ) was stored as a stock culture (ATG glycerol stock #845).

#### Verification of Expression of *T. thermophilus* $\beta$ by pA1-TN/AP1.L1

The pA1-TN plasmid was prepared and transformed into AP1.L1 bacteria (ATG glycerol stock #860, 861, 871). The bacterial growths of three isolates and isolation of total cellular protein were as described Example 2. A small aliquot (3  $\mu$ l) of supernatant from each of the three isolates was loaded onto a 4-20% SDS-polyacrylamide mini-gel (Novex, EC60255; 1 mm thick, with 15 wells/gel) in 25 mM Tris base, 192 mM glycine, and 0.1% SDS. The mini-gel was stained with Coomassie Blue. There were no visible protein bands from any of the isolates corresponding to the predicted migration region of  $\beta$ . Perhaps secondary structure of the high GC-*T. thermophilus* sequences was interfering with initiation. In an attempt to overcome this difficulty, we

- 136 -

constructed vectors with a gene encoding the native *T. thermophilus*  $\beta$  translationally coupled to the highly expressed *E. coli* *cca* gene.

5 Cloning *T. thermophilus* *dnaN* gene ( $\beta$ ) into a Translationally Coupled Vector pTAC-CCA-*ClaI*

To efficiently express  $\beta$  as a native protein we designed a vector to express  $\beta$  as a translationally coupled protein. As with expression of other *T. thermophilus* proteins in native form, our goal here is again to use translational coupling as described in Example #2. The *dnaN* gene was inserted behind the CCA adding enzyme and translationally coupled described for *T. thermophilus*  $\beta$ . First, the *dnaN* gene was amplified by using pA1-TN as a template by PCR. The forward/sense primer (ATG primer #P118-S78cla2, 5'-AGTCATCGATAATGAACATAACGGTTCCTCAAG AAA-3') (SEQ ID NO:59) has a *ClaI* restriction site in the non-complementary region. As in the cloning strategy developed for pTAC-CCA-TX, the non-complementary region also contains the "TA" of the stop (TAA) for the upstream CCA-adding protein fragment. The region of the primer complementary to the 5' end of the *T. thermophilus* *holA* gene begins with "A" which is the first nucleotide of the "ATG" start codon and the final "A" of the "TAA" stop codon. The reverse/antisense primer (ATG primer #P118-A1230spe, 5'-GAGGACTAGTCTACTAGACCCTGAGGGGCACCAC-3') (SEQ ID NO:60) contains a *SpeI* restriction site in the non-complementary portion of the primer and also an additional stop codon adjacent to the native stop codon, giving two stop codons in tandem. There was also a clamp region for efficient cutting with *SpeI*. Next, the PCR product was digested with *ClaI/SpeI* restriction enzymes and inserted into the pTAC-CCA-*ClaI* plasmid digested with the same enzymes. The plasmid was transformed into DH5 $\alpha$  bacteria and plasmids from ampicillin-resistant positive isolates were screened for by digestion with *ClaI/SpeI* restriction enzymes yielding 1.1 and 5.5 kb fragments. The sequence of both strands of the insert were verified by DNA sequencing (ATG SEQ #1749-1756; primers, P144-S23, P144-A1965, P118-

- 137 -

S290, P118-S639, P118-S1003, P118-A996, P118-A731, P118-A411). Sequence analysis confirmed the correct sequence was contained within the inserted region. This plasmid was named pTAC-CCA-TN and the isolate was stored as a stock culture (ATG glycerol stock #1074).

5

Verification of Expression of Native *T. thermophilus*  $\beta$ -Subunit by PTAC-CCA-TN/MGC1030 and pTAC-CCA-TN/AP1.L1

10 The pTAC-CCA-TN plasmid was prepared and transformed into MGC1030 bacteria (ATG glycerol stock #1087, 1088, 1089) and AP1.L1 (ATG glycerol stock #1090, 1091). The bacterial growths and isolation of total cellular protein were as described in Example #2. A small aliquot of each supernatant (3  $\mu$ l) containing total cellular protein was electrophoresised onto a 4-20% SDS-polyacrylamide mini-gel (Novex, EC60255; 1 mm thick, with 15 wells/gel) in 25 mM Tris base, 192 mM glycine, and 0.1% SDS. The mini-gels were stained with Coomassie Blue. A faint protein band corresponding to the predicted molecular mass of *T. thermophilus*  $\beta$  (40.5 kDa) was visualized slightly above the 40 kDa molecular weight standard of the Gibco 10 kDa protein ladder from the MGC1030 isolates, but could not be 20 discerned in the AP1.L1 isolates.

Large Scale Growth of pA1-CCA-TN/AP1.L1

25 Strain pA1-CCA-TN/AP1.L1 was grown in a 250 L fermentor (fermentor run #00-13), to produce cells for purification of *T. thermophilus*  $\beta$  as described in Example #2. Optimum induction times were determined as described in Example #2. Cell harvest was initiated 3 hours after induction, at OD<sub>600</sub> of 3.04, and the cells were chilled to 10°C during harvest. The harvest volume was 170 L, and the final harvest weight was approximately 0.9 kg of cell paste. An equal amount (w/w) of 50 mM Tris (pH 7.5) and 10% sucrose solution was added to the cell paste. Quality control results showed 10 out of 30 10 positive colonies on ampicillin-containing medium in the inoculum and

- 138 -

10/10 positive colonies at induction and 10/10 positive colonies at harvest. Cells were frozen by pouring the cells suspension into liquid nitrogen, and stored at -20°C, until processed.

5

#### EXAMPLE 7

##### Reconstitution of *T. thermophilus* DNA polymerase III holoenzyme

A primary goal of our endeavor has been to obtain the minimal assembly of the essential subunits of a processive thermophilic replicase that should permit processive synthesis of long stretches of DNA rapidly at elevated temperatures. We hypothesized that, minimally, *T. thermophilus*  $\alpha$ ,  $\beta$ , DnaX,  $\delta$  and  $\delta'$  would be required. With the availability of these proteins in N-terminal tagged forms, these proteins were used in an initial successful attempt at reconstitution. A modified form of the standard assay for the *E. coli* DNA polymerase III holoenzyme was used. The method comprised synthesis on a long single-stranded circular template primed by an RNA primer. M13 Gori single-stranded DNA was primed by the action of the *E. coli* DnaG primase in a large volume reaction that was aliquoted and frozen away for use in all reported assays. RNA primed M13 Gori single-stranded DNA is prepared (9.5 ml) by adding: 0.5 ml MgOAc (250 mM), 1.125 ml M13 Gori (240  $\mu$ M, nt), 0.2 ml purified *E. coli* SSB proteins (4.3 mg/ml), 1.5 ml dNTP mix (400  $\mu$ M dATP, dCTP, dGTP and 150  $\mu$ M [ $^3$ H]-dTTP (100 cpm/pmol), 0.5 ml rNTP mix (5 mM of each ATP, CTP, GTP and UTP), 0.025 ml purified *E. coli* primase (0.665 mg/ml) and 5.65 ml EDB (50 mM HEPES (pH 7.5), 20% glycerol, 0.02 % NP40, 0.2 mg/ml BSA). The radioactive dNTP mix was not used in the priming reaction but was used by the replication polymerase when it is added in the actual replication reaction (M13 Gori reaction). The priming mix was incubated at 30°C for 5 min and then placed on ice. The mixture was divided into 400  $\mu$ l aliquots and stored at -80°C until use. This mixture was used in all M13 Gori assays and is referred to as the primed-template mix.

- 139 -

Initially all of the purified *T. thermophilus* subunits (N-terminal tagged  $\alpha$ ,  $\beta$ , DnaX,  $\delta$  and  $\delta'$ ) were assayed together to determine if the complex could support processive polymerization of the M13 Gori primed template. The initial concentration of each *T. thermophilus* subunit used in this initial assay was arbitrarily set at 10 times the concentration of the *E. coli* Pol III subunits used in similar assays (Olson, *et al.*, *J. Biol. Chem.* 270:29570-29577 (1995)). The subunits were diluted in EDB buffer so that when combined (6  $\mu$ l total) and combined with 19  $\mu$ l of the primed-template mix to yield a 25  $\mu$ l reaction, the total levels of  $\alpha$ ,  $\beta$ ,  $\delta$ ,  $\delta'$  and DnaX were 1.25, 1.25, 1.0, 1.0 and 2.0 pmols, respectively (all subunit concentrations are as monomers). The reactions contained approximately 550 pmol of primed-template (total nucleotides). Reactions were initiated by combining the enzyme mix and the primed-template mix and incubating for 5 min at 50°C. The reactions were terminated by placing the reaction tubes on ice and adding 2 drops of 0.2 M NaPP<sub>i</sub> and 0.5 ml 10% TCA. The solution was filtered under vacuum through Whatman GF/C glass microfibre filters. The filters were then washed with 3 ml of 1M HCl/0.2 M NaPP<sub>i</sub> and 1 ml 95% EtOH and dried using a heat lamp. The pmol of nucleotides incorporated were quantified by scintillation counting. Other reactions were carried out, in which a different subunit was sequentially omitted from the reaction (FIG. 39). The final reaction (far right, FIG. 39) in which all subunits were present, but the concentration of  $\alpha$  was increased to 4.0 pmols. From the graph of these reactions in Fig. 39, when any subunit is omitted from the reaction the synthesis of DNA is decreased to very low levels, however, in the presence of all reactants maximum synthesis is observed. The results of these assays indicated that each subunit was functional and required for processive polymerization.

The optimum temperature for the M13 Gori assay using *T. thermophilus* Pol III subunits was determined. Reconstituted holoenzyme reactions were carried out as described above (using 4 pmol of  $\alpha$ ). The reactions were incubated for 5 min at the indicated temperatures. Results indicated that 50-65°C provided optimal temperatures for assaying *T.*



- 140 -

*thermophilus* replicative complex subunits (FIG. 40). Future assays will be carried out at 60°C.

The activity of each of the subunits used to reconstitute *T. thermophilus* holoenzyme was assessed individually in the presence of excess levels of the other four subunits. M13 Gori assays were initially designed so all of the subunits were present at the concentrations described in the experiment  $\beta$ ,  $\delta$ ,  $\delta'$  and DnaX were 1.25, 1.0, 1.0 and 2.0 pmols, respectively) except  $\alpha$ . The  $\alpha$ -subunit was added to different reactions in amounts varying from 0.125 to 4.2 pmol. Reactions were carried out at 60°C for 5 min. The results indicate that all available M13 Gori template has been replicated in the presence of 1 pmol of  $\alpha$  (FIG. 41A).

In the absence of the other subunits, there is a background level of non-processive synthesis catalyzed by  $\alpha$ . To define the background,  $\alpha$  was assayed in the M13 Gori reactions in varying amounts (0.125 to 8.4 pmol) (FIG. 42).

In the assay in which  $\alpha$  was titrated in M13 Gori holoenzyme assays, all the available template were replicated in the presence of 2 pmol of  $\alpha$ . In assays used to determine the background activity of  $\alpha$  at a concentration of 2 pmol, only 17 pmol nucleotide were incorporated. Therefore, all future assays will contain 2 pmol  $\alpha$ .

To determine the influence of  $\beta$  on the ability of the  $\alpha$  to processively replicate the primed-template,  $\alpha$  was assayed at varying amounts in the presence of all of the other subunits excluding  $\beta$  (FIG. 43). As can be seen when compared with the activity of  $\alpha$  alone (FIG. 42),  $\alpha$  was only slightly stimulated by the presence of the other holoenzyme subunits in the absence of the  $\beta$ -subunit.

As discussed above, translation of the *T. thermophilus dnaX* gene results in the expression of both  $\tau$ - and  $\gamma$ -subunits. The *dnaX* gene products in *E. coli* function as part of the clamp loading apparatus which catalyzes the assembly of the  $\beta$ -sliding clamp. The  $\tau$ -subunit also functions to dimerize Pol

- 141 -

III by direct contact with  $\alpha$  (Dallmann, H.G., and McHenry, C.S., *J. Biol. Chem.* 270:29563-29569 (1995)). From the Coomassie Blue stained gel (FIG. 10) of  $\tau$ - and  $\gamma$  it appears that approximately 60% expression is of the  $\gamma$ -subunit, while approximately 40% expression is of the  $\tau$ -subunit. Therefore, to determine the optimum amounts of  $\tau$ - and  $\gamma$  to use in the M13 Gori assays a wider range of concentrations were assayed (0.312 to 20 pmol). These assays are shown in FIG. 41B, and approximately 4 pmol of  $\tau$ - and  $\gamma$  are required to achieve maximum reconstitution of the replicative complex. In future M13 Gori assays, 4 pmol of DnaX will be used.

The  $\beta$ -subunit of replicative polymerases dramatically increased the processivity by linking the catalytic subunit to the DNA. To test the ability of  $\beta$  to reconstitute holoenzyme activity in the *T. thermophilus* system,  $\beta$  was titrated in the M13 Gori assay (0.08 to 10.0 pmol) (FIG. 41C). To insure maximum activity in following M13 Gori assays  $\beta$  will be present at 4 pmol.

Both  $\delta$  and the  $\delta'$  are constituents of the clamp loading complex in *E. coli* and likely serve a similar function in *T. thermophilus*. In *E. coli* they are both present in single copies and therefore smaller amounts may be needed to fully stimulate processive replications.

In future M13 Gori reactions,  $\delta$  and the  $\delta'$  will be at 2 pmol. These assays (FIGs. 41D and 41E) have allowed us to determine the concentration of all of the holoenzyme subunits required for optimal polymerization by the catalytic subunit ( $\alpha$ ). All of the subunits are required for processive polymerization. In the future, for purification of native subunits, assay conditions determined here will be used to follow each native protein through different purification steps. The assays will be designed so that the N-terminal tagged subunit corresponding to the native target subunit is omitted from the reaction mixes and aliquots from column elution fractions will be substituted. In this way fractions containing the target native protein will be detected.

- 142 -

## EXAMPLE 8

Protein-Protein Interactions Involving the Subunits of *T. thermophilus* Pol III

In view of considerable homology between DNA polymerase III genes of *E. coli* and *T. thermophilus*, we tested whether some of the known interactions of subunits in *E. coli* also occur in *T. thermophilus*. Gel filtration analysis of the interaction of Pol III subunits was performed using a Sephacryl<sup>®</sup> S-200 (Pharmacia Biotech) column (0.7 x 30 cm) equilibrated with HG.04 buffer. In all gel filtration experiments, the subunits (alone or in various combinations) were incubated at 60°C for 5 minutes (300 µl) prior to loading onto a Sephacryl S-200 column. The first three fractions (1 ml each) contained the void volume and all subsequent fractions contained 300 µl. Fractions were analyzed in 10% SDS-polyacrylamide gels stained with Commassie Brilliant Blue. The fractions were also analyzed in reconstitution activity assays in which all of the subunits were present as described in Example 7, except the subunit(s) being analyzed. In these reactions, 2 µl of each fraction was added to the reconstitution assay. If there was activity observed it was indicative of the presence of the subunit being analyzed in that fraction. All of these assays were carried out with N-terminal tagged proteins.

## Protein Interactions of Subunits Composing the Clamp-Loading Apparatus

In the *E. coli* clamp-loading complex,  $\delta$  and  $\delta'$  interact with each other and together with the DnaX subunits ( $\tau$  and  $\gamma$ ). Therefore, to determine if this interaction exists between the subunits composing the *T. thermophilus* clamp-loading complex we first carried out gel filtration experiments using  $\delta$ ,  $\delta'$ , and  $\tau/\gamma$  alone and in different combinations. Analysis of  $\delta$ ,  $\delta'$  and  $\tau/\gamma$  alone was performed using 200, 100 and 70 µg of protein, respectively. The elution profiles of the proteins assayed alone are shown in panel A, B and D of Figure 44.  $\delta$  eluted two fractions (fraction 18) before  $\delta'$  (fraction 20).  $\tau/\gamma$  likewise eluted two fractions (fraction 16) before  $\delta$ . The activity observed for

- 143 -

fractions in reconstitution assays are shown in the boxes beneath the SDS-polyacrylamide gels (Figure 44) and correspond to the fractions containing protein bands. Next,  $\delta$  (150  $\mu$ g) and  $\delta'$  (150  $\mu$ g) were assayed together to determine if an interaction occurs. A shift in the elution position would indicate an interaction between the tested subunits to form a larger complex than either subunit alone. In this assay (Figure 44, panel C),  $\delta$  and  $\delta'$  eluted two fraction earlier than  $\delta$  alone indicating an interaction between these two proteins was occurring. There is also a shift if the activity profile corroborating the protein elution profiles and further support an interaction between the two subunits. Unfortunately,  $\delta$  and  $\delta'$  are similar in size and could not be resolved on either 10% or on gradient polyacrylamide gels (data not shown). When  $\tau/\gamma$  (35  $\mu$ g) was assayed with  $\delta$  (70  $\mu$ g) and  $\delta'$  (85  $\mu$ g), the elution profile was shifted to fractions earlier than any of the subunits alone (Figure 44, panel E). The SDS-polyacrylamide gels indicate that all subunits are contained within the shifted fractions and the activity profile supports this observation.

To determine if the clamp-loading complex formed through interactions with either  $\delta$  or  $\delta'$  and  $\tau/\gamma$ , gel filtration experiments were carried out in which  $\tau/\gamma$  (60  $\mu$ g) and  $\delta$  (75  $\mu$ g) or  $\delta'$  (40  $\mu$ g) were analyzed. When  $\tau$  and  $\gamma/\delta$  were assayed together, no interaction was seen in either SDS-polyacrylamide gels or activity assays. However, when  $\tau/\gamma$  and  $\delta'$  were assayed together both DnaX proteins and  $\delta'$  were observed to be shifted together in both SDS-polyacrylamide gels and also in activity assays of the elution profile (data not shown). From these data, we theorize that the clamp-loader apparatus forms through interaction between  $\delta'$  and both  $\tau/\gamma$  and  $\delta$ .

#### Protein Interactions of the Catalytic Subunit $\alpha$ and Subunits Composing the Clamp-Loading Apparatus

In the *E. coli* Pol III holoenzyme, two  $\alpha$  catalytic subunits that replicate the leading and lagging DNA strands are held together by interactions with the DnaX protein  $\tau$ . (See, McHenry, C.S., J. Biol. Chem.,

- 144 -

257:2657-2663, [1982]). To determine if there are similar interactions occurring in the *T. thermophilus* holoenzyme the interaction of the  $\alpha$ -subunit with the DnaX proteins and other members of the clamp-loading apparatus were assayed by gel filtration. When  $\alpha$  (75 $\mu$ g) was subjected to gel filtration in the absence of other subunits the peak fraction eluted in fraction 16 (Figure 45, panel A). In the presence of  $\tau/\gamma$  (170  $\mu$ g), the elution is shifted to fraction 14 (Figure 45, panel B). From the previous section  $\tau/\gamma$  alone eluted in fraction 16 (Figure 44, panel D). These observations indicate that  $\alpha$  interacts with  $\tau/\gamma$  and probably through interactions with  $\tau$  since a larger relative amount of  $\tau$  appears to be shifted than  $\gamma$  when in the presence of  $\alpha$  (comparing Figure 44, panel D and Figure 45 panel B).

Next,  $\alpha$  (40  $\mu$ g),  $\tau/\gamma$  (115  $\mu$ g),  $\delta$  (50  $\mu$ g) and  $\delta'$  (50  $\mu$ g) are assayed together to determine if  $\delta$  and  $\delta'$  are also shifted in the presence of  $\alpha$  and  $\tau/\gamma$ . In these assays,  $\delta/\delta'$  appear to be shifted to fraction 14 (Figure 45, panel C) from fraction 18 when they are assayed together (Figure 44, panel C).

A dimer of  $\beta$  forms a ring structure that is loaded onto DNA and acts to tether the replicative complex to DNA during replication thereby construing the processivity characteristic to the Pol III holoenzyme in *E. coli*. In an attempt to determine the interaction of *T. thermophilus*  $\beta$  with other members of the *T. thermophilus* holoenzyme,  $\beta$  was assayed in gel filtration experiments. Initially,  $\beta$  was assayed alone (250  $\mu$ g, 20 $\mu$ M) to determine the elution profile in the absence of other proteins. The  $\beta$  subunit eluted from the Sephacryl S-200 column in fractions 12-20, suggesting the formation of large molecular weight multimers (Figure 46). To address the possibility that formation of multimers might be concentration dependent,  $\beta$  was re-assayed at a 10 fold lower concentration (25  $\mu$ g, 20 $\mu$ M). The results of these assays were identical to that seen at the higher concentration. Therefore, the interaction of  $\beta$  subunit with other components of the DNA polymerase III holoenzyme could not be examined by this method.

- 145 -

## EXAMPLE 9

Identification of *T. thermophilus* ssb gene

The ssb gene sequences from *A. aeolicus*, *B. subtilis*, *E. coli*, and *H. influenzae* was used to search the *T. thermophilus* genome database at Goettingen Genomics Laboratory. A sequence of a region of the *T. thermophilus* genome containing a putative *T. thermophilus* ssb gene was identified (using BLAST) and obtained (from Dr. Carsten Jacobi, Goettingen Genomics Laboratory, Institute of Microbiology and Genetics, Grisebachstrasse 8, Goettingen, Germany). Using the crude sequence, two PCR primers were designed to amplify the ssb gene. In the PCR reaction, the forward/sense primer (ATG primers P138-S540, 5'-GATCCATGGCTCGAGGCCTGAACCGC-3') (SEQ ID NO:29) was designed so that an *Nco*I site overlapped the start "ATG" codon. The reverse/antisense primer (P138-A1348, 5'-GACGGTACCTCATCAAACGGCAAATCCTC-3') (SEQ ID NO:30) was designed to add an additional "TGA" stop codon adjacent to the native "TGA" stop codon and a *Kpn*I restriction site in the non-complementary region. Both primers contained addition nucleotides to allow for efficient digestion with the *Nco*I and *Kpn*I restriction enzymes. The PCR reaction used *T. thermophilus* genomic DNA as a template and yielded a PCR product of 808 bp in length. This PCR fragment was inserted into pGEM-T Easy<sup>TM</sup> (Promega) vector per manufacturer directions. This plasmid was transformed into DH5 $\alpha$  bacteria and positive isolates were screened for by plasmid digestion with *Eco*RI restriction digest yielding 0.8 and 3.0 kb fragments. The plasmids from one positive isolate was selected and the correct sequence of both strands of the DNA were identified by DNA sequencing across the inserted region (ATG SEQ #1432-1436; primers, SP6, T7, P138-S913, P138-A1148, P138-A824). This plasmid was named pT-TSSB and the isolate was stored as a glycerol stock culture (ATG glycerol stock #838).

- 146 -

The DNA coding sequence of the *T. thermophilus* ssb gene (SEQ ID NO:31) in FIG. 47. The start codon (atg) and the stop codon (tga) are in bold print. Also shown below (FIG. 48) is the protein (amino acid) sequence (SEQ ID NO:32) derived from the DNA coding sequence.

5           The amino acid sequence of the *T. thermophilus* SSB protein was compared by sequence alignment with the sequence of several other SSB proteins (FIG. 49). The sequence of the *T. thermophilus* SSB protein was shown to contained an additional 50-70 amino acids in these comparisons. This is approximately 25% of the entire protein.

10           We know from previous studies that the SSB proteins from *E. coli* are functional in a homotetrameric form (Lowman and Ferrari, *Annu. Rev. Biochem.* 63:527-570 (1994)). The N-terminal 115 amino acids of the *E. coli* SSB contain the ssDNA-binding region. Other identified SSB proteins share similarities with *E. coli* SSB and contain the ssDNA binding region within the  
15           N-terminal region. These other SSB proteins are also thought to be active as tetramers. As shown (in FIG. 49) the *T. thermophilus* SSB contains an N-terminal region similar to the *E. coli* SSB and others, but is approximately 50% larger than the *E. coli* SSB. The sequence of the additional region (C-terminal region) of the *T. thermophilus* SSB was compared with its own N-  
20           terminal ssDNA binding regions (FIG. 50). Surprisingly, there was extensive sequence homology suggesting that this additional region may contain a second ssDNA binding region. If the *T. thermophilus* SSB contains two ssDNA binding regions it would be unique in SSB proteins yet studied and might explain the ability of *T. thermophilus* SSB to bind ssDNA at elevated  
25           temperatures.

Construction of Plasmid (pA1-TSSB) that Overexpresses *T. thermophilus* ssb gene (SSB) as a Native Protein

30           The TSSB gene contained an internal *KpnI* restriction site, therefore a partial *NcoI/KpnI* restriction digest allowed the entire *T. thermophilus* ssb gene to be extracted from the pT-TSSB plasmid. The *NcoI/KpnI* restriction

- 147 -

fragment containing the entire *T. thermophilus* ssb gene was inserted into the pA1-CB-NcoI plasmid digested with the same two restriction enzymes. The pA1-CB-NcoI plasmid contains a downstream hexahistidine and a biotinylation site, but it is downstream of the stop codon of the ssb gene and out of frame and will not be expressed. This plasmid was transformed into DH5 $\alpha$  bacteria and positive isolates were screened for by plasmid digestion with *NcoI/KpnI* restriction enzymes yielding 161 bp, 642 bp and 3.0 kb fragments. The plasmids from one positive isolate was selected and the correct sequence of the inserted DNA were confirmed by DNA sequencing across the inserted region (ATG SEQ #1445 and 1446; primers, P138-S5576, P138-S913). This plasmid was named pA1-TSSB and the isolate was stored as a glycerol stock culture (ATG glycerol stock #846).

#### Verification of Expression of Plasmid (pA1-TSSB) that Overexpresses *T. thermophilus* ssb gene as a Native Protein from pA1-TSSB/MGC1030

Plasmid pA1-TSSB was prepared from DH5 $\alpha$  bacteria as previously described. The plasmid was transformed into MGC1030 bacteria (ATG glycerol stock #872, 873, 874). The bacterial growths and isolation of total cellular protein were as described in Example 2. A small aliquot of supernatant (3  $\mu$ l) containing total cellular protein from each of the three isolates was loaded onto a 4-20% SDS-polyacrylamide mini-gel (Novex, EC60255; 1 mm thick, with 15 wells/gel) in 25 mM Tris base, 192 mM glycine, and 0.1% SDS. The mini-gel was stained with Coomassie Blue. There were no visible protein bands from any of the isolates corresponding to the predicted molecular weight of the *T. thermophilus* SSB.

#### Construction of a Plasmid (pA1-CB-TSSB) that Overexpress *T. thermophilus* SSB Gene Fused to a C-Terminal Peptide That Contains Hexahistidine and a Biotinylation Site

The gene encoding *T. thermophilus* SSB above was amplified by PCR using the pA1-TSSB plasmid as a template. The forward/sense primer (ATG



- 148 -

primer #P138-S540) was the same primer used in construction of pA1-TSSB and contained a region complementary to the 5' end of the *T. thermophilus* SSB gene. As before, a *NcoI* site overlapped the ATG start codon. The reverse/antisense primer was complementary to the 3' end of the *T.*  
5 *thermophilus* SSB gene excluding the stop codon (ATG primer #P138-A1343spe, 5'-GACGACTAGTAAACGGCAAATCCTCCTCC -3') (SEQ ID NO:33). This primer contained a *SpeI* restriction site adjacent to the complementary region of the primer. The *SpeI* site allowed for the expressed protein to contain two additional amino acids (Thr and Ser) between the C-  
10 terminal amino acid of the SSB protein and the C-terminal fusion peptide. This 800 bp PCR product was digested with *NcoI/SpeI* and inserted into the plasmid pA1-CB-*NcoI* digested with the same restriction enzymes as previously described. This plasmid was transformed into DH5 $\alpha$  bacteria and plasmids from positive isolates were screened for by digestion with *NcoI/SpeI*  
15 restriction enzymes yielding 0.8 and 5.6 kb fragments. One positive plasmid was selected and the sequence of the insert verified by DNA sequencing (ATG SEQ #1504-1507; primers, P38-S5576, P65-A106, P138-S913, P138-A1148). This plasmid was named pA1-CB-TSSB and the isolate was stored as a glycerol stock culture (ATG glycerol stock #897).

20  
Verification of Expression of *T. thermophilus* SSB Protein Fused to a C-Terminal Peptide That Contains Hexahistidine and a Biotinylation Site by pA1-CB-TSSB/MGC1030

25 The pA1-CB-TSSB plasmid was prepared and transformed into MGC1030 bacteria (ATG glycerol stock #919). The bacterial growths and isolation of total cellular protein were as described Example 2. A small aliquot of each supernatant (3  $\mu$ l) containing total cellular protein was loaded onto a 4-20% SDS-polyacrylamide mini-gel (Novex, EC60255; 1 mm thick,  
30 with 15 wells/gel) in 25 mM Tris base, 192 mM glycine, and 0.1% SDS. The mini-gels were stained with Coomassie Blue. The region of the gel in

- 149 -

which CB-TSSB was expected contained other intense protein bands and the *T. thermophilus* SSB protein could not be visualized.

Next, the total protein in each lysate was transferred (blotted) from polyacrylamide gel to nitrocellulose as described in Example 2. Each lane contained 1.5  $\mu$ l of the supernatant. Proteins on the blotted nitrocellulose were visualized by interactions with phosphatase-conjugated streptavidin as described above. The endogenous *E. coli* biotin binding protein, ~20 kDa was detectable in both induced and non-induced samples. A protein band corresponding to the *T. thermophilus* SSB protein migrated just below the 40 kDa molecular weight standard of the Gibco 10 kDa protein ladder. The predicted molecular weight of CB-TSSB is 33.5 kDa. This protein was observed as a faint band in the induced cultures, but was not observed in the uninduced control lysates.

#### Large Scale Growth of *T. thermophilus ssb* Gene Product Fused to a C-Terminal Peptide That Contains Hexahistidine and Biotinylation Site by pA1-CB-TSSB/MGC1030

Strain pA1-CB-TSSB/MGC1030 was grown in a 250 L fermentor to produce cells for purification of *T. thermophilus* SSB protein as described in Example 2. Cell harvest was initiated 3 hours after induction, at OD<sub>600</sub> of 8.4, and the cells were chilled to 10°C during harvest. The harvest volume was 178 L, and the final harvest weight was approximately 2.4 kg of cell paste. An equal amount (w/w) of 50 mM Tris (pH 7.5) and 10% sucrose solution was added to the cell paste. Quality control results showed 10 out of 10 positive colonies on ampicillin-containing medium in the inoculum, 8/10 positive colonies at induction and 8/10 positive colonies at harvest. Cells were frozen by pouring the cells suspension into liquid nitrogen, and stored at -20°C, until processed.

- 150 -

Determination of Optimal Ammonium Sulfate Precipitation Conditions of SSB Fused to a C-Terminal Peptide That Contains Hexahistidine and Biotinylation Site by pA1-CB-TSSB/MGC1030

5 Lysis was accomplished by creation of spheroplasts of the cells carrying the expressed *T. thermophilus* SSB proteins. First, from 100 g of a 1:1 suspension of frozen cells (50 g cells) in Tris-sucrose which had been stored at  $-20^{\circ}\text{C}$ , Fr I (170 ml, 14 mg/ml) was prepared. The preparation was as described in Example 2. The sample was then divided into 4 equal volumes  
10 (40 ml) and 6.56, 9.04, 11.64 and 14.44 g of ammonium sulfate (30%, 40%, 50% and 60% saturation) was added to each separate sample, respectively, over a 15 min interval at  $4^{\circ}\text{C}$ . The mixture rested for an additional 30 min at  $4^{\circ}\text{C}$  and was then centrifuged at  $23,000 \times g$  for 45 min at  $0^{\circ}\text{C}$ . The resulting pellets were resuspended in 2 ml Ni-NTA suspension buffer. The 30%, 40%,  
15 50% and 60% ammonium sulfate precipitated samples contained protein concentrations of 0.04, 0.18, 1.6 and 2.9 mg/ml, respectively. The samples were analyzed by SDS-polyacrylamide gel electrophoresis. The 30% and 40% ammonium sulfate precipitated samples contained no detectable SSB protein. The 50% and 60% samples contained bands of equal intensity of a protein  
20 migrating in the region corresponding to the molecular weight of *T. thermophilus* SSB. This band was faint compared to other proteins cited above and yields from large-scale preparations of the protein were thought to be small. Analysis by SDS-polyacrylamide gel electrophoresis of samples purified using Ni-NTA resin, but not ammonium sulfate precipitated also  
25 failed to allow a distinctive *T. thermophilus* protein band to be visualized.

Purification of *T. thermophilus* SSB Protein Fused to an C-Terminal Peptide That Contains Hexahistidine and a Biotinylation Site by pA1-CB-TSSB/MGC1030

30 Even though the initial analysis of expression levels of *T. thermophilus* SSB indicated low yields, enough protein could be isolated from large-scale preparations for antibody production. Lysis was accomplished by creation of

- 151 -

spheroplasts of the cells carrying the expressed *T. thermophilus* SSB. FrI (1270 ml, 10.6 mg/ml) was prepared from 800 g of a 1:1 suspension of frozen cells (400 g cells) stored in Tris-sucrose which had been stored at -20 °C as described in Example 2. To Fr I, ammonium sulfate (0.291 g to each initial ml  
5 Fraction I-50% saturation) was added over a 15 min interval. The mixture rested for an additional 30 min at 4°C and was then centrifuged at 23,000 x g for 45 min at 0°C. The resulting pellets were quick frozen by immersion in liquid nitrogen and stored at -80°C.

The protein pellets were resuspended in 150 ml of Ni<sup>++</sup>-NTA  
10 suspension buffer and homogenized using a Dounce homogenizer. The sample was clarified by centrifugation (16,000 x g) and the supernatant constituted Fr II (30 mg/ml). Fr II was added to 50 ml of a 50% slurry of Ni-NTA resin and rocked for 1.5 hours at 4°C. This slurry was then loaded onto a BioRad Econo-column (2.5 x 5 cm). The column was washed with 400 ml of  
15 Ni<sup>++</sup>-NTA wash buffer at a flow rate of 1.5 ml/min. *T. thermophilus* SSB was eluted in 250 ml of Ni<sup>++</sup>-NTA elution buffer containing a 10-200 mM imidazole gradient. The eluate was collected in 96 x 2.5 ml fractions. Fractions were subjected to SDS-polyacrylamide gel electrophoresis and biotin blot analysis, and fractions 28-70 were found to contain over 95% of  
20 total SSB protein (FIG. 51). *E. coli*  $\delta$  was used as a control since the molecular weight is similar to *T. thermophilus* SSB. In the Coomassie Blue stained gel, no clear protein bands corresponding to *T. thermophilus* SSB could be defined, however, the biotin blot analysis allowed us to determine fractions containing *T. thermophilus* SSB protein. Fractions 28-70 were  
25 pooled (100 ml, 0.76 mg/ml) and precipitated by addition of ammonium sulfate to 50% saturation. This sample was centrifuged as previously described resulting in two protein pellets.

- 152 -

Production of Polyclonal Antibodies Against *T. thermophilus* SSB Protein

One of the two *T. thermophilus* SSB precipitated protein pellets from above was resuspended in 20 ml of PBS and represented Fr III (1.5 mg/ml). A 2 ml UltraLink™ Immobilized Monomeric Avidin column (1.1 cm x 2.5 cm) (Pierce) was equilibrate in PBS plus 10% glycerol as per manufacturers instructions. The Fr III sample was loaded onto the avidin column, which was then washed (15 ml) and eluted (40 ml) in fractions as described in Example 3. The fractions were analyzed by SDS-polyacrylamide gel electrophoresis, and faint protein bands corresponding to *T. thermophilus* SSB could be detected in fractions 4-35. These fractions were pooled (27 ml, 0.01 mg/ml) and the protein was precipitated by adding ammonium sulfate to 50% saturation and the precipitate was collected by centrifugation (23,000 x g, 45 min, 0°C) and stored at -80°C. The pellet was then resuspended in 2 ml of PBS (0.01 mg/ml) and subjected to SDS-polyacrylamide gel electrophoresis and biotin blot analysis. This sample contained two faint upper molecular weight contaminating proteins, however because of the low yield of SSB protein, we decided to use this sample for antibody production.

The dialyzed samples were used to produce polyclonal antibodies against *T. thermophilus* ssb gene product (SSB protein) as described in Example 3.

Construction of a Plasmid (pA1-NB-TSSB) that Overexpress *T. thermophilus* ssb Gene Fused to an N-Terminal Peptide That Contains Hexahistidine and a Biotinylation Site

To increase expression of *T. thermophilus* SSB a vector was designed to express SSB as an N-terminal tagged protein. The forward/sense primer (ATG primer P138-S539pst, 5'-AAACTGCAGGCTCGAGGCCTGAA CCGCGTTTTCC-3') (SEQ ID NO:61) is designed so that the non-complementary portion contains a "AAA" clamp region and a *Pst*I site. The complementary portion of the primer is complementary to the first 25 nt of the

- 153 -

*ssb* gene beginning at codon 2, so that the first codon (the “ATG” start codon) is excluded. This will allow the PCR product to be inserted into the vector pA1-NB-AgeI at the *Pst*I site therefore fusing the *ssb* gene inframe with the N-terminal tagged peptide. The reverse/antisense primer (ATG primer P138-A1348stopspe, 5’-GACAACTAGTCATCAAAAACGGCAAATCCTCC-3’) (SEQ ID NO:62) contains a “GACA” clamp region and a *Spe*I restriction site in the non-complementary region. The non-complementary region also contains an additional “TGA” (TCA) stop codon that will be adjacent to the native “TGA” stop codon, giving two stop codons in tandem.

The PCR reaction used pA1-TSSB as a template and yielded a PCR product of 815 bp in length. This PCR fragment digested with *Pst*I and *Spe*I was inserted into pA1-NB-AgeI digested with *Pst*I and *Spe*I and resulted in the plasmid pA1-NB-TSSB which contained the entire gene encoding the *T. thermophilus* SSB. PA1-NB-TSSB was transformed into DH5 $\alpha$  bacteria and positive isolates were screened for by plasmid digestion with *Pst*I and *Spe*I restriction digest yielding 5.6 and 0.8 kb fragments. The plasmids from one positive isolate was selected and the correct sequence of both strands of the DNA were identified by DNA sequencing across the inserted region (ATG SEQ #1855-1859 and #1884-1885; primers: P138-S913, P138-A1148, P138-A824, NB-Sseq, p64-A215). This isolate was stored as a glycerol stock culture (ATG glycerol stock #1101).

Verification of Expression of *T. thermophilus* SSB Fused to an N-terminal Peptide that contains Hexahistidine and a Biotinylation Site

The pA1-NB-TSSB plasmid was prepared and transformed into MGC1030 (ATG glycerol stock #1128) and AP1.L1 bacteria (ATG glycerol stock #1129). Three isolates from each transformation were selected for farther study. The bacterial growths and isolation of total cellular protein were as described in Example #2. A small aliquot of supernatant (3  $\mu$ l) containing total cellular protein from each of the three isolates was loaded onto a 4-20% SDS-polyacrylamide mini-gel (Novex, EC60255; 1 mm thick, with 15

- 154 -

wells/gel) in 25 mM Tris base, 192 mM glycine, and 0.1% SDS. The mini-gel was stained with Coomassie Blue. Distinct protein bands from all of the isolates corresponding to the predicted migration region of *T. thermophilus* SSB (approximately 33.5 kDa) were visualized.

5           Next, the total protein in each lysate was transferred (blotted) from polyacrylamide gel to nitrocellulose as described in Example #2. Each lane contained 1.5 ul of the supernatant containing total protein. Proteins on the blotted nitrocellulose were visualized by interactions with phosphatase-conjugated streptavidin as described above. The endogenous *E. coli* biotin  
10 binding protein, ~20 kDa was detectable in both induced and non-induced samples. A protein band corresponding to the *T. thermophilus* SSB protein migrated midway between the 30 and 40 kDa molecular weight standard of the Gibco 10 kDa protein ladder. This protein was observed as a very intense band in the induced cultures, but was not observed in the uninduced control lysates.

15           Optimization of Expression of *T. thermophilus* SSB by pA1-NB-TSSB

          Since expression of *T. thermophilus ssb* gene yielded low or no detectable proteins when expressed as both a native or coupled to an C-  
20 terminal fusion peptide, extra care was taken with *T. thermophilus* SSB linked to an N-terminal fusion peptide to achieve optimum expression. Expression was analyzed using both *E. coli* strains MGC1030 and AP1.L1 carrying pA1-NB-TSSB at different induction times. Growth of bacterial cultures and analysis were carried out as described in Example #2. Biotin blot analysis  
25 indicated that expression levels were higher at 37 °C and also slightly better when expressed in the AP1.L1 bacterial strain. The optimum yield of *T. thermophilus* SSB was attained by 3 h post induction and at 37 °C; this induction time will be used in subsequent experiments.

- 155 -

## Large Scale Growth of pA1-NB-TSSB/AP1.L1

Strain pA1-NB-TSSB/AP1.L1 was grown in a 250 L fermentor to produce cells for purification of *T. thermophilus* SSB fused to an N-terminal peptide that contains hexahistidine and biotinylation site as described in Example #2. Cell harvest was initiated 3 hours after induction at  $OD_{600} = 5.0$ , and the cells were chilled to 10 °C during harvest. The harvest volume was 180 L, and the final harvest weight was approximately 2.07 kg of cell paste. An equal amount (w/w) of 50 mM Tris-HCl (pH 7.5) and 10% sucrose solution was used to resuspend the cell paste. Cells were frozen by pouring the cell suspension into liquid nitrogen, and stored at -20 °C until processed. Quality control results showed 10 out of 10 positive colonies on ampicillin-containing medium in the inoculum, 10 out of 10 positive colonies at induction and 10 out of 10 positive colonies at harvest.

Purification of *T. thermophilus* ssb Product Fused to an N-Terminal Peptide That Contains Hexahistidine and a Biotinylation Site

Lysis of 800 g of a 1:1 suspension of frozen cells (400 g of cells) containing pA1-NB-TSSB stored in Tris-sucrose at -20 °C was preformed as described Example #2. The recovered supernatant (1.4 l) constituted Fr I (10.7 mg/ml). To Fr I, ammonium sulfate (0.291 g to each initial ml Fraction I-50% saturation) was added over a 15 min interval. The mixture was stirred for an additional 30 min at 4 °C and the precipitate was collected by centrifugation (23,000 x g, 45 min, 0 °C). The resulting pellets were quick frozen by immersion in liquid nitrogen and stored at -80 °C.

The pellets from Fr I were resuspended in 100 ml of  $Ni^{++}$ -NTA suspension buffer (50 mM Tris-HCl (pH 7.5), 40 mM KCl, 7 mM  $MgCl_2$ , 10% glycerol, 7 mM  $\beta$ ME, 0.1 mM PMSF) and homogenized using a Dounce homogenizer. The sample was clarified by centrifugation (16,000 x g) and the supernatant constituted Fr II. Fr II was added to 40 ml of a 50% slurry of  $Ni^{++}$ -NTA resin in  $Ni^{++}$ -NTA suspension buffer and rocked for 1.5 hours at 4



- 156 -

°C. This slurry was then loaded onto a BioRad Econo-column (2.5 x 5 cm). The column was washed with 300 ml of Ni<sup>++</sup>-NTA wash buffer (50 mM Tris-HCl (pH 7.5), 1 M KCl, 7 mM MgCl<sub>2</sub>, 10% glycerol, 10 mM Imidazole, 7 mM βME) at a flow rate of 0.5 ml/min. The protein was eluted in 300 ml of Ni<sup>++</sup>-NTA elution buffer (50 mM Tris-HCl (pH 7.5), 40 mM KCl, 7 mM MgCl<sub>2</sub>, 10% glycerol, 7 mM βME) containing a 10-200 mM imidazole-HCl (pH 7.5) gradient. The *T. thermophilus* SSB eluted across the second half of the gradient and contained a number of contaminating proteins as determined by SDS-polyacrylamide gels. These fractions were pooled and the protein isolated by precipitation with ammonium sulfate (0.291 g to each initial ml of sample-50% saturation).

One-third of the precipitated protein was resuspended in 20 ml of PBS containing 10% glycerol and further purified using a monomeric avidin column as describe in Example #3. The yield from this column was almost homologous *T. thermophilus* SSB (20 ml, 0.23 mg/ml).

The remaining two-thirds of the precipitated protein was resuspended in 20 ml of Ni<sup>++</sup>-NTA suspension buffer, mixed with 10 ml of a 50% slurry of Ni<sup>++</sup>NTA resin and rocked for 1.5 hours at 4 °C. The resin was poured into a column and purified as before. The yield from this column was also almost homologous *T. thermophilus* SSB (68 ml, 0.5 mg/ml). Both protein purifications were frozen by imersion in liquid nitrogen and stored at -80 °C for future analysis.

#### Cloning *T. thermophilus ssb* Gene (SSB) into a Translationally Coupled Vector pTAC-CCA-ClaI

To efficiently express SSB as a native protein we designed a vector to express SSB as a translationally coupled protein. We again use translational coupling as described in Example 2. The *T. thermophilus ssb* gene was inserted behind the CCA adding enzyme and translationally coupled as described for the other *T. thermophilus* proteins expressed by translationally coupling. First, the *ssb* gene was amplified by using pA1-TSSB as a template

- 157 -

by PCR. The forward/sense primer (ATG primer #P138-S533cla2, 5'-  
ACTGATCGATAATGGCTCGAGGCCTGAACCGC-3') (SEQ ID NO:63)  
has a *Cla*I restriction site in the non-complementary region. The non-  
complementary region also contains the "TA" of the stop (TAA) for the  
upstream CCA-adding protein fragment. The region of the primer  
complementary to the 5' end of the *T. thermophilus* *holA* gene begins with "A"  
which is the first nucleotide of the "ATG" start codon and the final "A" of the  
"TAA" stop codon. The reverse/antisense primer (ATG primer #P138-  
A1348stopspe, 5'-GACAACTAGTCATCAAAACGGCAAATCCTCC-3')  
(SEQ ID NO:64) contains a *Spe*I restriction site in the non-complementary  
portion of the primer and also an additional stop codon adjacent to the native  
stop codon, giving two stop codons in tandem. There was also a clamp region  
for efficient cutting with *Spe*I. Next, the PCR product was digested with  
*Cla*I/*Spe*I restriction enzymes and inserted into the pTAC-CCA-*Cla*I plasmid  
digested with the same enzymes. The plasmid was transformed into DH5 $\alpha$   
bacteria and plasmids from ampicillin-resistant positive isolates were screened  
for by digestion with *Cla*I/*Spe*I restriction enzymes yielding 0.8 and 5.5 kb  
fragments. The sequence of both strands of the insert were verified by DNA  
sequencing (ATG SEQ #1688-1692, 1721; primers, P144-S23, P144-A1965,  
P65-A106, P138-S913, P138-A1148, P138-A828). Sequence analysis  
confirmed that the correct sequence was contained within the inserted region.  
This plasmid was named pTAC-CCA-TSSB and the isolate was stored as a  
stock culture (ATG glycerol stock #1033).

Verification of Expression of Native *T. thermophilus* SSB by PTAC-CCA-  
TSSB/MGC1030 and pTAC-CCA-TSSB/AP1.L1

The pTAC-CCA-TSSB plasmid was prepared and transformed into  
MGC1030 bacteria (ATG glycerol stock #1071, 1072, 1073) and AP1.L1  
(ATG glycerol stock #1079). The bacterial growths and isolation of total  
cellular protein were as described in Example 2. A small aliquot of each  
supernatant (3  $\mu$ l) containing total cellular protein was electrophoresised onto

- 158 -

a 4-20% SDS-polyacrylamide mini-gel (Novex, EC60255; 1 mm thick, with 15 wells/gel) in 25 mM Tris base, 192 mM glycine, and 0.1% SDS. The mini-gels were stained with Coomassie Blue. A faint protein band corresponding to the predicted molecular mass of *T. thermophilus* SSB (29.8 kDa) was visualized migrating just above the 30 kDa molecular weight standard of the Gibco 10 kDa protein ladder in the AP1.L1 isolates.

#### EXAMPLE 10

##### Identification of Two *T. thermophilus* *dnaQ* genes ( $\epsilon$ -subunits)

From the previous *Tth* patent application (U.S. Application #09/151888), the probe 5'-CCT CGA ACA CCT CCT GCC GCA AGA CCC TTC GAC CCA-3' (SEQ ID NO:34) was used to screen a lambda library containing *T. thermophilus* genomic DNA. Using this probe, over 100 strong positive plaques were identified and verified by replating. Three were grown up and the DNA purified as described for *dnaE*. One (cl#5.1.1) was selected for further sequencing. The sequence of a major portion of the *dnaQ* gene was obtained by direct sequencing of the insert in the isolated lambda DNA using sequences selected from the PCR product to initiate sequencing. As previously described (U.S. Application # 09/151888), upon preliminary examination of the sequence, it was found to encode one continuous open reading frame (ORF) that showed significant homology to other DNA polymerase III  $\epsilon$ -subunits from other bacteria (based on a BLAST search). A strong secondary structure or other block prevented obtaining more 3' sequence of the *T. thermophilus* *dnaQ* gene.

The *T. thermophilus* genome database at Goettingen Genomics Laboratory was searched for using the sequence of the ORF identified above. It indicated two open reading frames that showed close similarity to our partial *Tth* *dnaQ* sequence. The closest match was designated *dnaQ*-1 and the poorer-scoring match *dnaQ*-2. *DnaQ*2 is described in Example 14. Only homology scores, not the actual sequence data was available from the web

- 159 -

site. Dr. Carsten Jacobi (Goettingen Genomics Laboratory, Institute of Microbiology and Genetics, Grisebachstrasse 8, Goettingen, Germany) agreed to provide crude, unannotated incomplete sequence information in the regions of our BLAST hits on their website. Examination of the sequences indicated two clearly independent but homologous *dnaQ*-like genes. Initially, focusing on *dnaQ*-1, several restriction sites (*Ngo*MIV, *Bam*HI, *Nco*I and *Sac*I) were identified upstream and downstream of the *dnaQ*-1 gene. To obtain the entire correct sequence of the *T. thermophilus dnaQ*-1 gene, the lambda clone #5.1.1 (described in U.S. Application # 09/151888) was digested with *Ngo*MIV, *Bam*HI, *Nco*I and *Sac*I restriction enzymes. Restriction digest was carried out on 5 µl (approx. 2.8 µg DNA) of the lambda clone #5.1.1. The digested DNA samples were electrophoresised on a 1% agarose gel and transferred by capillary transfer to MSI Magnagraph nylon membrane. The blot (10 x 15 cm) was treated with 20 ml of Ambion Ultrahyb™ hybridization solution at 42°C for 2 h, then 20 ng of the biotinylated probe (probe 5'-CCT CGA ACA CCT CCT GCC GCA AGA CCC TTC GAC CCA-3' (SEQ ID NO:35) was added to the hybridization bag and the blot was incubated at 42°C overnight. The blot was processed and detected using an NEB Phototope CDP-Star chemiluminescence detection kit per manufacturer's instructions (New England BioLabs). The probe hybridized with a 1 kb *Ngo*MIV/*Sac*I restriction fragment. This 1 kb *Ngo*MIV/*Sac*I restriction fragment was chosen for subcloning and sequencing.

A pUC21 cloning vector (Sigma) was chosen as the recipient DNA, and was subjected to *Ngo*MIV/*Sac*I digestion. The *Ngo*MIV/*Sac*I fragment of the lambda clone #5.1.1 was ligated into the digested pUC21. The resulting plasmid was transformed into DH5α and isolates were selected for by ampicillin-resistance. Plasmids were purified from one isolate and screened by *Ngo*MIV/*Sac*I and *Xho*I digestion of plasmids yielding the expected 1.0 and 2.7 kb and 480 bp and 3.3 kb fragments, respectively. Both DNA strands of the inserted region were sequenced (ATG SEQ #1437-1442; primers, M13 reverse primer, P140-S839, P140-S1209, P140-A1443, P140-A1089 and

- 160 -

pUC21-A829). This plasmid was named pUC21-TQ and the isolate was stored as a stock culture (ATG glycerol stock #843).

The DNA coding sequence of the *T. thermophilus dnaQ-1* gene (SEQ ID:NO:36) is shown in FIG. 52. The start codon (gtg) and the stop codon (tga) are in bold print. Also shown in FIG. 53 is the protein (amino acid) sequence (SEQ ID NO:37) derived from the DNA coding sequence.

#### Construction of Plasmid (pA1-TQ) that Expresses *T. thermophilus dnaQ-1* gene

Expression of *T. thermophilus dnaQ-1* gene product ( $\epsilon$ 1-subunit) as a native protein was accomplished. The construction of pA1-TQ was performed by insertion of the native *T. thermophilus dnaQ-1* gene into the pA1-CB-Cla-2 plasmid. The pUC21-TQ plasmid was prepared and the *T. thermophilus dnaQ-1* gene was amplified out of the pUC21-TQ plasmid using PCR. The forward/sense primer (ATG primer #P140-S96cla; 5'-CCATCGATGCCTGCAGGTCTGGAGG-3') (SEQ ID NO:38) used in the PCR reaction was designed to have an upstream *Cla*I site that overlaps the AT of the ATG start codon used for the *dnaQ-1* gene. The native start codon for the *dnaQ-1* gene is GTG, this has been replaced in the primer with an ATG start codon to allow for expression in *E. coli*. The reverse/antisense primer (ATG primer #P140-A713kpn; 5'-GACGGTACCTCATCAGTACCTGAGCCGGGCCAA-3') (SEQ ID NO:39) was designed to have an additional stop codon placed in tandem with the native stop codon. This additional stop codon was adjacent to a *Kpn*I restriction site in the non-complementary region of the primer. The PCR product was digested with *Cla*I and *Kpn*I restriction enzymes. The digested PCR product was inserted into the *Cla*I/*Kpn*I digested pA1-CB-Cla-2 plasmid. These plasmids were transformed into DH5 $\alpha$  bacteria and positive isolates were selected by ampicillin-resistance. Plasmids were purified from one clone and screened by *Cla*I/*Kpn*I digest of purified plasmids yielding 0.6 and 5.6 kb fragments. The inserted region in this plasmid was subjected to DNA sequencing to confirm the correct sequence

- 161 -

(ATG SEQ #1508-1511; primers, P38-S5576, P65-A106, P140-S839 and P140-A1089). This plasmid was named pA1-TQ and the isolate was stored as a stock culture (ATG glycerol stock #900).

5 Verification of Expression of Plasmid (pA1-TQ) that Overexpresses *T. thermophilus* dnaQ-1 gene ( $\epsilon$ -Subunit) as a Native Protein from pA1-TQ/MGC1030

10 The pA1-TQ plasmid was prepared and transformed into MGC1030 bacteria. Three isolates were selected (ATG glycerol stock #921, 922, 923) for further study. The bacterial growths and isolation of total cellular protein were as described in Example 2. A small aliquot (3  $\mu$ l) of supernatant containing total cellular protein from each of the three isolates, was loaded onto a 4-20% SDS-polyacrylamide mini-gel (Novex, EC60255; 1 mm thick, 15 wells/gel) in 25 mM Tris base, 192 mM glycine, and 0.1% SDS. The mini-gel was stained with Coomassie Blue. There were no visible protein bands from any of the isolates corresponding to the predicted migration region of the  $\epsilon$ -subunit.

20 Construction of a Plasmid (pA1-CB-TQ) that Overexpress *T. thermophilus* dnaQ-1 ( $\epsilon$ 1-subunit) Fused to a C-Terminal Peptide That Contains Hexahistidine and a Biotinylation Site

25 Since initial attempts to express the native  $\epsilon$ -subunit failed, a vector was designed to couple the *T. thermophilus* dnaQ-1 gene to a fusion peptide containing a hexahistidine and a biotinylation site. The construction of pA1-CB-TQ was also performed by insertion of the *T. thermophilus* dnaQ-1 gene into the pA1-CB-Cla-2 plasmid. The reverse/antisense primer however was designed to add a *Spe*I site onto the 3' end of the gene allowing insertion into 30 the pA1-CB-Cla-2 plasmid in frame with the DNA encoding the C-terminal peptide that contains hexahistidine and a biotinylation site. The pUC21-TQ plasmid was prepared for use as the PCR template. The *T. thermophilus* dnaQ-1 gene was amplified out of the pUC21-TQ plasmid using PCR. The

- 162 -

forward/sense primer (ATG primer # P140-S96cla) was the same as used in producing pA1-TQ. The reverse/antisense primer (ATG primer #P140-A708Spe; 5'-CCTCACTAGTGTACCTGAGCCGGGCCAA-3') (SEQ ID NO:40) was designed so that a *SpeI* restriction site was adjacent to the penultimate codon (the stop codons were excluded). The *SpeI* site allowed for the expressed protein to contain two additional amino acids (Thr and Ser) between the C-terminal amino acid of the  $\epsilon$ -subunit and the C-terminal fusion peptide. The PCR product was digested with *ClaI* and *SpeI* restriction enzymes and inserted into the *ClaI/SpeI* digested pA1-CB-Cla-2 plasmid. The plasmid was then transformed into DH5 $\alpha$  bacteria and plasmids from positive isolates were selected by ampicillin-resistance. Plasmids were isolated from one positive isolate and screened by digestion with *ClaI* and *SpeI* restriction enzymes yielding 0.6 and 5.6 kb fragments. The correct sequence of the inserted region was confirmed by DNA sequencing (ATG SEQ #1526-1529; primers, P38-S5576, P65-A106, P140-S839 and P140-A1089). This plasmid was named pA1-CB-TQ and the isolate was stored as a stock culture (ATG glycerol stock #911).

Verification of Expression of Plasmid (pA1-CB-TQ1) that Overexpresses *T. thermophilus* dnaQ-1 gene ( $\epsilon$ 1-Subunit) Fused to a C-Terminal Peptide That Contains Hexahistidine and a Biotinylation Site from pA1-CB-TQ/MGC1030

The pA1-CB-TQ1 plasmid was prepared and transformed into MGC1030 bacteria. Three isolate was selected (ATG glycerol stock #929) for further study. The bacterial growths and isolation of total cellular protein were as described in Example 2. A small aliquot (3  $\mu$ l) of supernatant containing total cellular protein from each of the three isolates was electrophoresised onto a 4-20% SDS-polyacrylamide mini-gel (Novex, EC60255; 1 mm thick, with 15 wells/gel) in 25 mM Tris base, 192 mM glycine, and 0.1% SDS. The mini-gel was stained with Coomassie Blue. There were no protein bands from any of the isolates corresponding to the predicted migration region of the  $\epsilon$ 1-subunit.

- 163 -

Next, the total protein from the lysate was transferred (blotted) from polyacrylamide gel to nitrocellulose as described in Example 2. Proteins on the blotted nitrocellulose were visualized by interactions with phosphatase-conjugated streptavidin. The endogenous *E. coli* biotin-CCP protein, ~20 kDa was detectable in both induced and non-induced samples. A protein band corresponding to the  $\epsilon$ 1-subunit migrated approximately midway between the 20 and 30 molecular weight standards of the Gibco 10 kDa protein ladder. This is consistent with the expected molecular weight of 25.8 kDa. This protein was observed as a faint band in the induced cultures, but was not observed in the uninduced control in lysates from the AP1.L1 strain. The protein was expressed at levels too low to justify purification attempts.

#### EXAMPLE 11

##### *T. thermophilus* UvrD Helicase

##### Identification and Cloning *T. thermophilus* *uvrD* Gene

The UvrD protein sequence from *E. coli* was used to search the *T. thermophilus* genome database at Goettingen Genomics Laboratory. The region of the *T. thermophilus* genome (2-4-2000 contig working.0.15372, region 40201-46740) containing a putative *T. thermophilus* *uvrD* gene was identified (using BLAST) and obtained (from Dr. Carsten Jacobi, Goettingen Genomics Laboratory, Institute of Microbiology and Genetics, Grisebachstrasse 8, Goettingen, Germany). Using the crude sequence, two PCR primers were designed to amplify the *uvrD* gene. All of the vectors that we have for expressing N-terminal tagged proteins require a *Pst*I site for insertion of the 5' end of the gene into the vectors. However, there is a *Pst*I site within the *uvrD* gene. To overcome this problem a *Nsi*I site was added to the non-complementary portion of the forward/sense primer (ATG primer P159-S1689, 5'-GACTATGCATAGCGACGCCCTCCTAGCCCCCTCAAC-3') (SEQ ID NO:65). The *Nsi*I restriction cut site of the PCR product will leave a



- 164 -

four nucleotide overhang (TGCA) that can be utilized (annealed) by a *Pst*I restriction cut site on the pA1-NB-AgeI plasmid. The *Pst*I and the *Nsi*I site will be destroyed by the ligation, but the *uvrD* gene will be inserted inframe with the DNA encoding the N-terminal fusion peptide. The PCR product will  
5 exclude the GTG start codon and begins at codon 2, with the *Nsi*I site adjacent to codon 2. The reverse/antisense primer (ATG primer P159-A3786, 5'-GACTACTAGTCTATCATGCCGGCTTAAGCTCCGCG-3') (SEQ ID NO:66) was designed to add an additional "TAG" stop codon adjacent to the native "TGA" stop codon and a *Spe*I restriction site in the non-complementary  
10 region. Both primers contained addition nucleotides to allow for efficient digestion with the *Nsi*I and *Spe*I restriction enzymes. The PCR reaction used *T. thermophilus* genomic DNA as a template and yielded a PCR product of 2410 bp in length. This PCR fragment digested with *Nsi*I and *Spe*I was inserted into pA1-NB-AgeI digested with *Pst*I and *Spe*I and resulted in the  
15 plasmid pA1-NB-TuvrD which contained the entire gene encoding the *T. thermophilus* UvrD helicase.

PA1-NB-TuvrD was transformed into DH5 $\alpha$  bacteria and positive isolates were screened for by plasmid digestion with *Ndi*I and *Spe*I restriction digest yielding 5.5 and 2.5 kb fragments. The plasmids from one positive  
20 isolate was selected and the correct sequence of both strands of the DNA were identified by DNA sequencing across the inserted region (ATG SEQ #1993-2005; primers: P159-S1926, P159-S2326, P159-S2733, P159-S3134, P159-S3540, P159-A3592, P159-A3332, P159-A3154, P159-A2770, P159-A2471, P159-A2060, NB-Sseq, p64-A215). This isolate was stored as a glycerol stock  
25 culture (ATG glycerol stock #1161).

Upon comparing this DNA sequence with the crude sequence obtained from the *T. thermophilus* genome database at Goettingen Genomics Laboratory several discrepancies were observed. Therefore, to confirm the  
30 sequence of the DNA encoding the *uvrD* gene obtained by sequencing the inserted region of this isolate a second clone was sequenced in the critical areas (ATG SEQ #2007-2008, primers: P159-A3154 and P159-A2471). The

- 165 -

changes observed by sequencing the gene from those reported for the crude DNA sequence were: C>G at position 337 (no amino acid change); C>T at position 466 (no amino acid change); G deletion at position 731 (frameshift); G insertion at position 776 (frameshift); T>C at position 1474 (no amino acid change); T>C at position 1475 (Ser>Pro amino acid change); G>C at position 1481 (Pro>Ala amino acid change).

The DNA coding sequence of the *T. thermophilus* *uvrD* gene is shown (FIG. 54, SEQ ID NO:67). The start codon (gtg) and the stop codon (tga) are in bold print. Also shown is the protein (amino acid) sequence (FIG. 55, SEQ ID NO:68) derived from the DNA coding sequence.

#### Verification of Expression of *T. thermophilus* UvrD Fused to an N-terminal Peptide that contains Hexahistidine and a Biotinylation Site

The pA1-NB-TuvrD plasmid was prepared and transformed into MGC1030 and AP1.L1 bacteria. Three isolates from each transformation were selected for further study. The bacterial growths and isolation of total cellular protein were as described in Example 2. A small aliquot of supernatant (3 µl) containing total cellular protein from each of the three isolates was loaded onto a 4-20% SDS-polyacrylamide mini-gel (Novex, EC60255; 1 mm thick, with 15 wells/gel) in 25 mM Tris base, 192 mM glycine, and 0.1% SDS. The mini-gel was stained with Coomassie Blue. There were no protein bands from any of the isolates corresponding to the predicted migration region of *uvrD* (approximately 80 kDa).

Next, the total protein in each lysate was transferred (blotted) from polyacrylamide gel to nitrocellulose as described in Example 2. Each lane contained 1.5 µl of the supernatant. Protein bands on the blotted nitrocellulose were visualized by interactions with phosphatase-conjugated streptavidin as described above. The endogenous *E. coli* biotin-CCP protein, ~20 kDa was detectable in both induced and non-induced samples. A protein band corresponding to the *T. thermophilus* UvrD protein migrated just below the 80 kDa molecular weight standard of the Gibco 10 kDa protein ladder. This

- 166 -

protein was observed as a faint band in the induced cultures, but was not observed in the uninduced control lysates. The glycerol stocks of pA1-NB-TuvrD in MGC1030 and AP1.L1 (ATG glycerol stock #1177 and 1178, respectively) were stored at -80 °C.

5

## EXAMPLE 12

### *T. thermophilus* DnaG - Primase

#### Identification and Cloning *T. thermophilus* dnaG Gene

10

The DnaG protein sequence from *E. coli* was used to search the *T. thermophilus* genome database at Goettingen Genomics Laboratory. The region of the *T. thermophilus* genome (2-4-2000 contig working.0.24624, region 42961-48060) containing a putative *T. thermophilus* dnaG gene was identified (using BLAST) and obtained (from Dr. Carsten Jacobi, Goettingen Genomics Laboratory, Institute of Microbiology and Genetics, Grisebachstrasse 8, Goettingen, Germany). Using the crude sequence, two PCR primers were designed to amplify the dnaG gene. The forward/sense primer (ATG primer P161-S1922, 5'-GACTCTGCAGGACGCGGGCC AGGCGGTGGAGCTGA-3') (SEQ ID NO:69) is designed so that the non-complementary portion contains a "GACT" clamp region and a *Pst*I site. The complementary portion of the primer is complementary to the first 25 nt of the dnaG gene beginning at codon 2, so that the first codon (the "ATG" start codon) is excluded. This will allow the PCR product to be inserted into the vector pA1-NB-Avr2(BamH1-) at the *Pst*I site therefore fusing the gene inframe with the N-terminal tagged peptide. The reverse/antisense primer (ATG primer P161-A3714, 5'-GACTACTAGTCTACTAGGTGGACCAG CCCGAAGGA-3') (SEQ ID NO:70) contains a "GACT" clamp region and a *Spe*I restriction site in the non-complementary region. The non-complementary region also contains an additional "TAG" (CTA) stop codon

15

20

25

30

- 167 -

that will be adjacent to the native "TAG" stop codon, giving two stop codons in tandem.

The sequence for the *T. thermophilus* *dnaG* gene is (FIG. 56, SEQ ID NO:71). The start (atg) and the stop (tga) are shown as bold. Also shown is the protein (amino acid) sequence derived from the DNA coding sequence (FIG. 57, SEQ ID NO:72).

The PCR reaction used *T. thermophilus* genomic DNA as a template and yielded a PCR product of 2148 bp in length. This PCR fragment digested with *Pst*I and *Spe*I was inserted into pA1-NB-Avr2(BamHI-) digested with *Pst*I and *Spe*I and resulted in the plasmid pA1-NB-TdnaG which contained the entire gene encoding the *T. thermophilus* DnaG primase. PA1-NB-TdnaG was transformed into DH5 $\alpha$  bacteria and positive isolates were screened for by plasmid digestion with *Pst*I and *Spe*I restriction digest yielding 5.6 and 2.15 kb fragments. The plasmids from one positive isolate was selected and the correct sequence of both strands of the DNA were identified by DNA sequencing across the inserted region (ATG SEQ #2022-2031; primers: P161-S2260, P161-S2650, P161-S3056, P161-S3349, P161-A3375, P161-A3048, P161-A2694, P161-A2389, NB-Sseq, p64-A215). The DNA sequence determined here was compared to the crude sequence from Goettingen Genomics Laboratory and no changes were observed. This isolate was stored as a glycerol stock culture (ATG glycerol stock #1173).

#### Verification of Expression of *T. thermophilus* DnaG Fused to an N-Terminal Peptide that contains Hexahistidine and a Biotinylation Site

The pA1-NB-TdnaG plasmid was prepared and transformed into MGC1030 and AP1.L1 bacteria. Three isolates from each transformation were selected for farther study. The bacterial growths and isolation of total cellular protein were as described Example 2. A small aliquot of supernatant (3  $\mu$ l) containing total cellular protein from each of the three isolates was loaded onto a 4-20% SDS-polyacrylamide mini-gel (Novex, EC60255; 1 mm thick, with 15 wells/gel) in 25 mM in Tris base, 192 mM glycine, and 0.1% SDS.

- 168 -

The mini-gel was stained with Coomassie Blue. Distinct protein bands from all of the isolates corresponding to the predicted migration region of DnaG (approximately 80 kDa) were visualized.

Next, the total protein in each lysate was transferred (blotted) from polyacrylamide gel to nitrocellulose as described in Example 2. Each lane contained 1.5 ul of the supernatant. Proteins on the blotted nitrocellulose were visualized by interactions with phosphatase-conjugated streptavidin. The endogenous *E. coli* biotin-CCP protein, ~20 kDa was detectable in both induced and non-induced samples. A protein band corresponding to the *T. thermophilus* DnaG protein migrated midway between the 70 and 80 kDa molecular weight standard of the Gibco 10 kDa protein ladder. This protein was observed as a very intense band in the induced cultures, but was not observed in the uninduced control lysates. The glycerol stocks of pA1-NB-TdnaG in MGC1030 and AP1.L1 (ATG glycerol stock #1182 and 1183, respectively) were stored at -80 °C.

### EXAMPLE 13

#### *T. thermophilus* PriA - Helicase

##### Identification and Cloning *T. thermophilus* priA Gene

The PriA protein sequence from *E. coli* was used to search the *T. thermophilus* genome database at Goettingen Genomics Laboratory. The region of the *T. thermophilus* genome (2-4-2000 contig working.0.2196, region 36541-42840) containing a putative *T. thermophilus* priA gene was identified (using BLAST) and obtained (from Dr. Carsten Jacobi, Goettingen Genomics Laboratory, Institute of Microbiology and Genetics, Grisebachstrasse 8, Goettingen, Germany). Unsure of the crude sequence and proper placement of the start and stop codons we decided to sequence the region beginning approximately 200 bp upstream of the putative start codon to approximately 200 bp downstream of the putative stop codon. Using the crude

- 169 -

sequence, two PCR primers were designed to amplify the *priA* gene. The forward/sense primer (ATG primer P162-S963, 5'-CCGAAGAGCCTCTCCAGGAGGGGGAGGAGGGGAACCA-3') (SEQ ID NO:73) and the reverse/antisense primer (ATG primer P162-A3625, 5'-GGGGCAGCCGCAAGGGGTAAGGGTAGAAAA-3') (SEQ ID NO:74) using *T. thermophilus* genomic DNA as a substrate yielded a 2676 bp DNA fragment. This DNA fragment was inserted into the T/A cloning site of pGEM-TEasy plasmid per manufacturer instructions creating pT-TpriA. This plasmid was transformed into DH5 $\alpha$  bacteria and positive isolates were screened for by plasmid digestion with *EcoRI* restriction digest yielding 2.7 and 3.0 kb fragments and digestion with *HinDIII* yielding 0.6 and 5.1 kb fragments. The plasmids from one positive isolate was selected and the sequence of both strands of the DNA were identified by DNA sequencing across the inserted region (ATG SEQ #1969-1982, 2009-2017, and 2042-2043; primers: SP6, T7-Seq2, P162-S1292, P162-S1656, P162-S2026, P162-S2408, P162-S2781, P162-S3173, P162-A3257, P162-A2825, P162-A2446, P162-A2038, P162-A1709, P162-A1243, P162-S963, P162-A1335, P162-S1146). The sequence obtained here was compared to the crude sequence from Goettingen Genomics Laboratory and no discrepancies were discerned. This isolate was stored as a glycerol stock culture (ATG glycerol stock #1155).

The sequence for the *T. thermophilus priA* gene is shown (FIG. 58, SEQ ID NO:75). The start (gtg) and the stop (tag) are shown as bold. Also shown is the protein (amino acid) sequence (FIG. 59, SEQ ID NO:76) derived from the DNA coding sequence.

To insert the *T. thermophilus* gene into the expression vector pA1-NB-AgeI to be expressed as an N-terminal tagged protein a 5' *PstI* restriction site and a 3' *SpeI* restriction site was needed. This was accomplished by PCR amplifying the *T. thermophilus* gene using the forward/sense primer (ATG primer P162-S1052, 5'-GACTCTGCAGCGGGTGCTTCAGGTGGCCCTTC-3') (SEQ ID NO:77) designed so that the non-complementary portion contains a "GACT" clamp region and a *PstI* restriction site. The complementary

- 170 -

portion of the primer is complementary to the first 22 nt of the *priA* gene beginning at codon 2, so that the first codon (the start codon in this case is "GTG") is excluded. The reverse/antisense primer (ATG primer P162-A3180, 5'-CAGTACTAGTCTAGTCCTCCAAAAGCCCCACGA-3') (SEQ ID NO:78) contains a "CAGT" clamp region and a *SpeI* restriction site in the non-complementary region. This PCR primer can not contain an additional stop codon or it will create an additional *SpeI* site that will be adjacent to the native "TAG" (cta). The PCR reaction used pT-TpriA as a template and yielded a PCR product of 2130 bp in length. This PCR fragment was digested with *PstI* and *SpeI* was inserted into pA1-NB-AgeI digested with *PstI* and *SpeI* and resulted in the plasmid pA1-NB-TpriA which contained the entire gene encoding the *T. thermophilus* PriA helicase. pA1-NB-TpriA was transformed into DH5 $\alpha$  bacteria and positive isolates were screened for by plasmid digestion with *PstI* and *SpeI* restriction digest yielding 5.6 and 2.13 kb fragments. The plasmids from one positive isolate was selected and the correct sequence of both strands of the DNA were identified by DNA sequencing across the inserted region (ATG SEQ #2057-2070; primers: P162-S1146, P162-S1292, P162-S1656, P162-S2026, P162-S2408, P162-S2781, P162-A2825, P162-A2446, P162-A2038, P162-A1709, P162-A1335, P162-A1243, NB-Sseq, p64-A215). This isolate was stored as a glycerol stock culture (ATG glycerol stock #1192).

#### Verification of Expression of *T. thermophilus* PriA Fused to an N-Terminal Peptide that contains Hexahistidine and a Biotinylation Site

The pA1-NB-TpriA plasmid was prepared and transformed into MGC1030 and AP1.L1 bacteria. Three isolates from each transformation were selected for farther study. The bacterial growths and isolation of total cellular protein were as described in Example 2. A small aliquot of supernatant (3  $\mu$ l) containing total cellular protein from each of the three isolates was loaded onto a 4-20% SDS-polyacrylamide mini-gel (Novex, EC60255; 1 mm thick, with 15 wells/gel) in 25 mM in Tris base, 192 mM glycine, and 0.1% SDS.

- 171 -

The mini-gel was stained with Coomassie Blue. Distinct protein bands from all of the isolates corresponding to the predicted migration region of PriA (approximately 81.5 kDa) were visualized.

Next, the total protein in each lysate was transferred (blotted) from polyacrylamide gel to nitrocellulose as described in Example 2. Each lane contained 1.5 ul of the supernatant. The endogenous *E. coli* biotin-CCP protein, ~20 kDa was detectable in both induced and non-induced samples. A protein band corresponding to the *T. thermophilus* PriA protein migrated midway between the 80 and 90 kDa molecular weight standard of the Gibco 10 kDa protein ladder. This protein was observed as a very intense band in the induced cultures, but was not observed in the uninduced control lysates. The glycerol stocks of pA1-NB-TpriA in MGC1030 and AP1.L1 (ATG glycerol stock #1196 and 1197, respectively) were stored at -80 °C.

#### EXAMPLE 14

##### Cloning *T. thermophilus* dnaQ-2

The ORF encoding *T. thermophilus* dnaQ-2 gene contained two possible start sites that were out of frame with each other. Therefore to determine the correct start codon and to confirm the sequence the gene encoding the *T. thermophilus* dnaQ-2 gene from *T. thermophilus*, genomic DNA was amplified by PCR using two primers located approximately 200 bp upstream and downstream of the start and stop codon. Using a forward/sense primer (ATG primer #P133-S150, 5'-TGGGGGCGAACCTCACG-3') (SEQ ID NO: 79) and a reverse/antisense primer (ATG primer #P133-A1237, 5'-ACCCCGGCCTTCCAGTCCA-3')(SEQ ID NO: 80) and *T. thermophilus* genomic DNA as a substrate resulted in a 1088 bp PCR product. This PCR fragment was inserted into a pGEM-T Easy plasmid and transformed into DH5α and isolates were selected for by ampicillin-resistance. Plasmids were purified from one isolate and screened by *Eco*RI digestion of plasmids yielding the expected 1.1 and 3.0 kb fragments. Both DNA strands of the



- 172 -

inserted region were sequenced (ATG SEQ #1330-1335; primers, SP6, T7, P133-S456, P133-S894, P133-A896, P133-A527). There was a one base pair discrepancy with the DNA sequence when compared with the crude sequence. There were four "T"s shown in the crude sequence beginning 61  
 5 bases downstream of the first GTG start codon. The DNA sequencing by ATG, Inc. indicated and confirmed only three "T"s. This indicated that both possible GTG start codons were in frame and that the first GTG was likely the native start codon. This plasmid was named pT-TQ2 and the isolate was stored as a stock culture (ATG glycerol stock #785).

10 The DNA coding sequence of the *T. thermophilus dnaQ-1* gene (SEQ ID:NO:81) is shown in FIG. 60. The two possible start codons (gtg) and the stop codon (tga) are in bold print. Also shown in FIG. 61 is the protein (amino acid) sequence (SEQ ID NO:82) derived from the DNA coding sequence.

#### 15 Construction of a Plasmid (pA1-TQ2) that Expresses *T. thermophilus dnaQ-2* gene

Expression of *T. thermophilus dnaQ-2* gene product ( $\epsilon 2$ -subunit) as a native protein was accomplished. The construction of pA1-TQ2 was  
 20 performed by insertion of the native *T. thermophilus dnaQ-2* gene into the pA1-CB-NcoI plasmid. The *T. thermophilus dnaQ-2* gene was amplified out of *T. thermophilus* genomic DNA using PCR. The forward/sense primer (ATG primer #P133-S442nco; 5-  
 GGATCCATGGAGCGGGTGGTGCGGCCCTTCTG-3) (SEQ ID NO:83)  
 25 used in the PCR reaction was designed to have an upstream *NcoI* site that overlaps the TGG of the ATG start codon used for the *dnaQ-2* gene. The native start codon for the *dnaQ-2* gene is GTG, this has been replaced in the primer with an ATG start codon to allow for expression in *E. coli*. The reverse/antisense primer (ATG primer #P133-A109kpn; 5-  
 30 AAGCTAGGTACCTACTACCTCCCGAGTTCCCAAAG-3) (SEQ ID NO:84) was designed to have an additional stop codon placed in tandem with the native stop codon. This additional stop codon was adjacent to a *KpnI*

- 173 -

restriction site in the non-complementary region of the primer. The PCR product was digested with *Nco*I and *Kpn*I restriction enzymes. The digested PCR product was inserted into the *Nco*I/*Kpn*I digested pA1-CB-*Nco*I plasmid. These plasmids were transformed into DH5 $\alpha$  bacteria and positive isolates were selected by ampicillin-resistance. Plasmids were purified from one clone and screened by *Nco*I/*Kpn*I digest of purified plasmids yielding 0.65 and 5.7 kb fragments. The inserted region in this plasmid was subjected to DNA sequencing to confirm the correct sequence (ATG SEQ #1384-1387, 1404-1405; primers, P38-S5576, P65-A106, P133-S635, and P133-A817). This plasmid was named pA1-TQ2 and the isolate was stored as a stock culture (ATG glycerol stock #815).

Verification of Expression of Plasmid (pA1-TQ2) that Overexpresses *T. thermophilus* *dnaQ*-2 gene ( $\epsilon$ 2-Subunit) as a Native Protein from pA1-TQ2/MGC1030 and pA1-TQ2/AP1.L1

The pA1-TQ2 plasmid was prepared and transformed into MGC1030 and AP1.L1 bacteria. Three isolates were selected from pA1-TQ2/MGC1030 (ATG glycerol stock #828, 829, 830) and from pA1-TQ2/AP1.L1 (ATG glycerol stock #847, 848, 849) for further study. The bacterial growths and isolation of total cellular protein were as described in Example 2. A small aliquot (3  $\mu$ l) of supernatant containing total cellular protein from each of the six isolates, was loaded onto a 4-20% SDS-polyacrylamide mini-gel (Novex, EC60255; 1 mm thick, with 15 wells/gel) in 25 mM Tris base, 192 mM glycine, and 0.1% SDS. The mini-gel was stained with Coomassie Blue. There were no protein bands that could be resolved from surrounding endogenous *E. coli* from any of the isolates corresponding to the predicted migration region of the  $\epsilon$ 2-subunit.

- 174 -

Construction of a Plasmid (pA1-CB-TQ2) that Overexpress *T. thermophilus* *dnaQ*-2 ( $\epsilon$ 2-subunit) Fused to a C-Terminal Peptide That Contains Hexahistidine and a Biotinylation Site

5                Since initial attempts to express the native  $\epsilon$ -subunit failed, a vector was designed to couple the *T. thermophilus dnaQ*-2 gene to a fusion peptide containing a hexahistidine and a biotinylation site. The construction of pA1-CB-TQ2 was also performed by insertion of the *T. thermophilus dnaQ*-2 gene into the pA1-CB-NcoI plasmid. The forward/sense primer was the same used  
10 in construction of pA1-TQ2 (ATG primer #P133-S442nco). The *T. thermophilus* genomic DNA was used as the PCR template. The reverse/antisense primer (ATG primer #P133-A1084Spe; 5-CCTCACTAGTCCTCCCGAGTTCCCAAAGCGT-3) (SEQ ID NO:85) was designed so that a *Spe*I restriction site was adjacent to the penultimate codon  
15 (the stop codon was excluded). The *Spe*I site allowed for the expressed protein to contain two additional amino acids (Thr and Ser) between the C-terminal amino acid of the  $\epsilon$ 2-subunit and the C-terminal fusion peptide. The PCR product was digested with *Nco*I and *Spe*I restriction enzymes and inserted into the *Nco*I/*Spe*I digested pA1-CB-NcoI plasmid. The plasmid was  
20 then transformed into DH5 $\alpha$  bacteria and plasmids from positive isolates were selected by ampicillin-resistance. Plasmids were isolated from one positive isolate and screened by digestion with *Nco*I and *Spe*I restriction enzymes yielding 0.65 and 5.7 kb fragments. The correct sequence of the inserted region was confirmed by DNA sequencing (ATG SEQ #1388-1391, 1406-  
25 1407; primers, P38-S5576, P65-A106, P133-S635 and P133-A817). This plasmid was named pA1-CB-TQ2 and the isolate was stored as a stock culture (ATG glycerol stock #816).

- 175 -

### Verification of Expression of Plasmid (pA1-CB-TQ) that Overexpresses *T. thermophilus* dnaQ-2 gene(ε2-Subunit) Fused to a C-Terminal Peptide That Contains Hexahistidine and a Biotinylation Site from pA1-CB-TQ/MGC1030

The pA1-CB-TQ2 plasmid was prepared and transformed into MGC1030 and AP1.L1 bacteria. Three isolate was selected from pA1-CB-TQ2/MGC1030 (ATG glycerol stock #831, 832, 833) and from pA1-CB-TQ2/AP1.L1 (ATG glycerol stock #850,851, 852) for further study. The bacterial growths and isolation of total cellular protein were as described in Example 2. A small aliquot (3  $\mu$ l) of supernatant containing total cellular protein from each of the six isolates was electrophoresed onto a 4-20% SDS-polyacrylamide mini-gel (Novex, EC60255; 1 mm thick, with 15 wells/gel) in 25 mM in Tris base, 192 mM glycine, and 0.1% SDS. The mini-gel was stained with Coomassie Blue. There were no protein bands from any of the isolates that could be resolved from endogenous *E. coli* proteins in the region corresponding to the predicted migration region of the  $\epsilon$ 2-subunit.

Next, the total protein from the lysate was transferred (blotted) from polyacrylamide gel to nitrocellulose as described in Example 2. Proteins on the blotted nitrocellulose were visualized by interactions with phosphatase-conjugated streptavidin. The endogenous *E. coli* biotin-CCP protein, ~20 kDa was detectable in both induced and non-induced samples. A protein band corresponding to the  $\epsilon 2$ -subunit could not be detected. The protein was expressed at levels too low to justify purification attempts.

\* \* \* \* \*

Having now fully described the present invention in some detail by way of illustration and example for purposes of clarity of understanding, it will be obvious to one of ordinary skill in the art that same can be performed by modifying or changing the invention with a wide and equivalent range of conditions, formulations and other parameters thereof, and that such

- 176 -

modifications or changes are intended to be encompassed within the scope of the appended claims.

5 All publications, patents and patent applications mentioned in this specification are indicative of the level of skill of those skilled in the art to which this invention pertains, and are herein incorporated by reference to the same extent as if each individual publication, patent or patent application was specifically and individually indicated to be incorporated by reference.

- 177 -

WHAT IS CLAIMED IS:

1. An isolated polypeptide wherein said polypeptide comprises an amino acid sequence having at least 95% sequence identity to the amino acid sequence of SEQ ID NO: (uvrD helicase) 68.

2. The polypeptide of claim 1 wherein said polypeptide has the amino acid sequence of SEQ ID NO: 68.

3. An isolated polynucleotide molecule comprising a nucleotide sequence encoding a polypeptide of claim 1.

4. The isolated polynucleotide molecule of claim 3 comprising a nucleic acid having the sequence of SEQ ID NO: 67.

5. A vector comprising an isolated polynucleotide of claim 3.

6. A host cell comprising a vector of claim 5.

7. The isolated polypeptide of claim 1 wherein said polypeptide is a uvrD helicase from a thermophilic organism.

8. The isolated polypeptide of claim 7 wherein said thermophilic organism is *Thermus thermophilus*.

9. An isolated polypeptide wherein said polypeptide comprises an amino acid sequence having at least 95% sequence identity to the amino acid sequence of SEQ ID NO: (DNA-G Primase) 72.

10. The polypeptide of claim 9 wherein said polypeptide has the amino acid sequence of SEQ ID NO: 72.

- 178 -

11. An isolated polynucleotide molecule comprising a nucleotide sequence encoding a polypeptide of claim 9.

5 12. The isolated polynucleotide molecule of claim 11 comprising a nucleotide sequence having the sequence of SEQ ID NO: 71.

13. A vector comprising an isolated polynucleotide of claim 11.

10 14. A host cell comprising a vector of claim 13.

15 15. The isolated polypeptide of claim 9 wherein said polypeptide is a DNA G primase from a thermophilic organism.

16 16. The isolated polypeptide of claim 15 wherein said thermophilic organism is *Thermus thermophilus*.

20 17. An isolated polypeptide wherein said polypeptide comprises an amino acid sequence having at least 95% sequence identity to the amino acid sequence of SEQ ID NO: (priA helicase) 76.

18. The polypeptide of claim 17 wherein said polypeptide has the amino acid sequence of SEQ ID NO: 76.

25 19. An isolated polynucleotide molecule comprising a nucleotide sequence encoding a polypeptide of claim 17.

20. The isolated polynucleotide molecule of claim 19 comprising a nucleotide sequence having the sequence of SEQ ID NO: 75.

30

21. A vector comprising an isolated polynucleotide of claim 19.

- 179 -

22. A host cell comprising a vector of claim 21.

5           23. The isolated polypeptide of claim 17 wherein said polypeptide  
is a priA helicase from a thermophilic organism.

24. The isolated polypeptide of claim 23 wherein said thermophilic  
organism is *Thermus thermophilus*.

10           25. An isolated polypeptide wherein said polypeptide comprises an  
amino acid sequence having at least 95% sequence identity to the amino acid  
sequence of SEQ ID NO: (delta subunit) 10.

15           26. The polypeptide of claim 25 wherein said polypeptide has the  
amino acid sequence of SEQ ID NO: 10.

27. An isolated polynucleotide molecule comprising a nucleotide  
sequence encoding a polypeptide of claim 25.

20           28. The isolated polynucleotide molecule of claim 27 comprising a  
nucleotide sequence having the sequence of SEQ ID NO: 9.

29. A vector comprising an isolated polynucleotide of claim 27.

25           30. A host cell comprising a vector of claim 29.

31. The isolated polypeptide of claim 25 wherein said polypeptide  
is a delta subunit from a thermophilic organism.

30           32. The isolated polypeptide of claim 31 wherein said thermophilic  
organism is *Thermus thermophilus*.



- 180 -

33. An isolated antibody molecule, where in said antibody specifically binds to at least one antigenic determinant on a polypeptide of claim 25.

5

34. An isolated polypeptide wherein said polypeptide comprises an amino acid sequence having at least 95% sequence identity to the amino acid sequence of SEQ ID NO: (delta prime subunit) 17.

10

35. The polypeptide of claim 34 wherein said polypeptide has the amino acid sequence of SEQ ID NO: 17.

36. An isolated polynucleotide molecule comprising a nucleotide sequence encoding a polypeptide of claim 34.

15

37. The isolated polynucleotide molecule of claim 36 comprising a nucleotide sequence having the sequence of SEQ ID NO: 16.

38. A vector comprising an isolated polynucleotide of claim 36.

20

39. A host cell comprising a vector of claim 38.

40. The isolated polypeptide of claim 34 wherein said polypeptide is a delta prime subunit from a thermophilic organism.

25

41. The isolated polypeptide of claim 40 wherein said thermophilic organism is *Thermus thermophilus*.

42. An isolated antibody molecule, wherein said antibody specifically binds to at least one antigenic determinant on a polypeptide of claim 37.

30

- 181 -

43. An isolated polypeptide wherein said polypeptide comprises an amino acid sequence having at least 95% sequence identity to the amino acid sequence of SEQ ID NO: (beta subunit) 23.

5           44. The polypeptide of claim 43 wherein said polypeptide has the amino acid sequence of SEQ ID NO: 23.

45. An isolated polynucleotide molecule comprising a nucleotide sequence encoding a polypeptide of claim 43.

10

46. The isolated polynucleotide molecule of claim 45 comprising a nucleotide sequence having the sequence of SEQ ID NO: 22.

47. A vector comprising an isolated polynucleotide of claim 45.

15

48. A host cell comprising a vector of claim 47.

49. The isolated polypeptide of claim 43 wherein said polypeptide is a beta subunit from a thermophilic organism.

20

50. The isolated polypeptide of claim 49 wherein said thermophilic organism is *Thermus thermophilus*.

51. An isolated antibody molecule, wherein said antibody specifically binds to at least one antigenic determinant on a polypeptide of claim 43.

25

52. An isolated polypeptide wherein said polypeptide comprises an amino acid sequence having at least 95% sequence identity to the amino acid sequence of SEQ ID NO: (ssb protein) 32.

30

- 182 -

53. The polypeptide of claim 52 wherein said polypeptide has the amino acid sequence of SEQ ID NO: 32.

54. An isolated polynucleotide molecule comprising a nucleotide  
5 sequence encoding a polypeptide of claim 52.

55. The isolated polynucleotide molecule of claim 54 comprising a nucleotide sequence having the sequence of SEQ ID NO: 31.

10 56. A vector comprising an isolated polynucleotide of claim 54.

57. A host cell comprising a vector of claim 56.

15 58. The isolated polypeptide of claim 52 wherein said polypeptide is an SSB protein from a thermophilic organism.

59. The isolated polypeptide of claim 58 wherein said thermophilic organism is *Thermus thermophilus*.

20 60. An isolated antibody molecule, where in said antibody specifically binds to at least one antigenic determinant on a polypeptide of claim 55.

25 61. An isolated polypeptide wherein said polypeptide comprises an amino acid sequence having at least 95% sequence identity to the amino acid sequence of SEQ ID NO: (epsilon1, dnaQ-1) 37.

62. The polypeptide of claim 61 wherein said polypeptide has the amino acid sequence of SEQ ID NO: 37.

- 183 -

63. An isolated polynucleotide molecule comprising a nucleotide sequence encoding a polypeptide of claim 61.

5 64. The isolated polynucleotide molecule of claim 63 comprising a nucleotide sequence having the sequence of SEQ ID NO: 36.

65. A vector comprising an isolated polynucleotide of claim 63.

66. A host cell comprising a vector of claim 65.

10 67. The isolated polypeptide of claim 61 wherein said polypeptide is an epsilon subunit from a thermophilic organism.

15 68. The isolated polypeptide of claim 67 wherein said thermophilic organism is *Thermus thermophilus*.

69. An isolated antibody molecule, where in said antibody specifically binds to at least one antigenic determinant on a polypeptide of claim 61.

20 70. An isolated polypeptide wherein said polypeptide comprises an amino acid sequence having at least 95% sequence identity to the amino acid sequence of SEQ ID NO: (dnaQ-2 protein) 82.

25 71. The polypeptide of claim 70 wherein said polypeptide has the amino acid sequence of SEQ ID NO: 82.

72. An isolated polynucleotide molecule comprising a nucleotide sequence encoding a polypeptide of claim 70.

30

- 184 -

73. The isolated polynucleotide molecule of claim 72 comprising a nucleotide sequence having the sequence of SEQ ID NO: 81.

74. A vector comprising an isolated polynucleotide of claim 72.

75. A host cell comprising a vector of claim 74.

76. The isolated polypeptide of claim 70 wherein said polypeptide is an epsilon-2 subunit from a thermophilic organism.

77. The isolated polypeptide of claim 76 wherein said thermophilic organism is *Thermus thermophilus*.

78. An isolated antibody molecule, where in said antibody specifically binds to at least one antigenic determinant on a polypeptide of claim 73.

79. A method of producing a polypeptide encoded by a nucleotide sequence, wherein said polypeptide comprises an amino acid sequence having at least 95% sequence identity to the amino acid sequence of one of SEQ ID NOS: 68, 72, 76, 10, 17, 23, 32, 37, and 82, comprising culturing a host cell comprising said nucleotide sequence under conditions such that said polypeptide is expressed, and recovering said polypeptide.

80. A method of synthesizing DNA which comprises utilizing one or more polypeptides, said one or more polypeptides comprising an amino acid sequence having at least 95% sequence identity to an amino acid sequence selected from the group consisting of SEQ ID NOS: 68, 72, 76, 10, 17, 23, 32, 37 and 82.

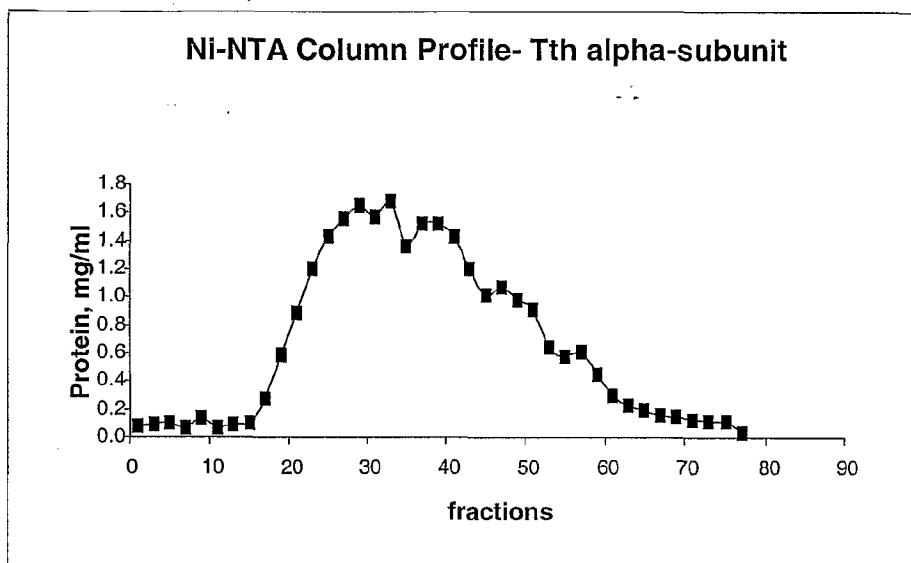
- 185 -

81. The method of claim 80 further comprising providing in any order: a reaction mixture comprising components comprising template, and nucleotides, and incubating said reaction mixture for a length of time and at a temperature sufficient to obtain DNA synthesis.

5

82. The method of claim 81 wherein said one or more polypeptides further comprises an N-terminal linked peptide or a C-terminal linked peptide.

1/63

**FIG. 1**

Column profile of Ni<sup>++</sup>-NTA purification of *T. thermophilus*  $\alpha$ -Subunit

2/63

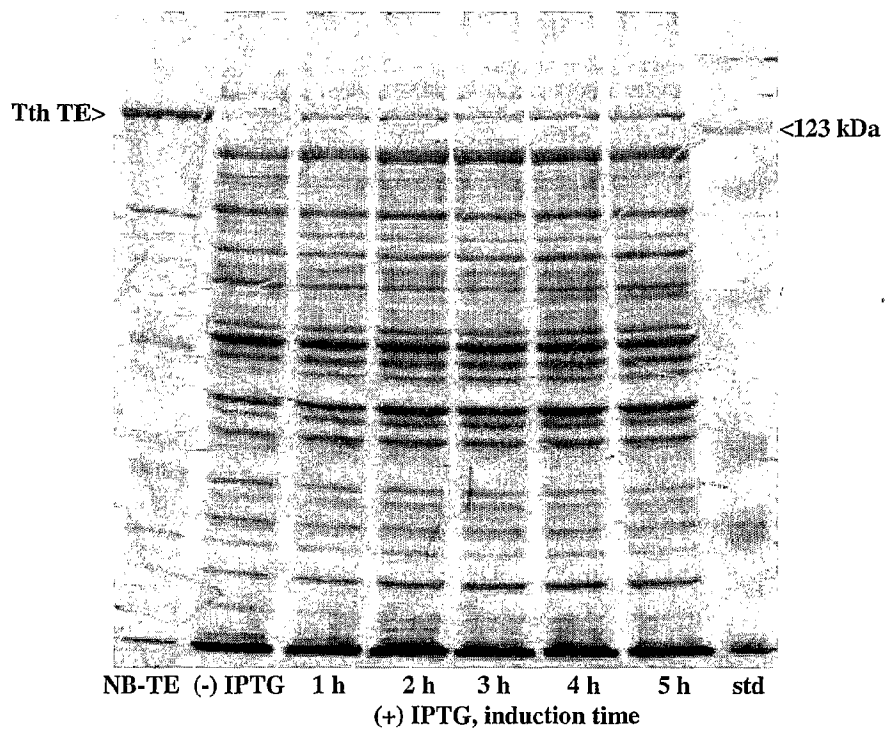
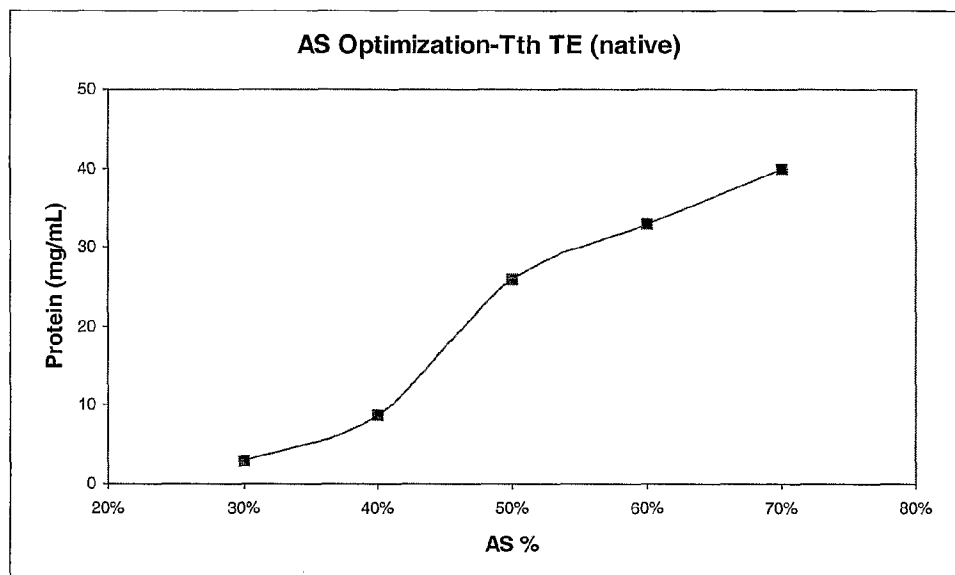


FIG. 2

Optimization of expression of pTAC-CCA-TE.

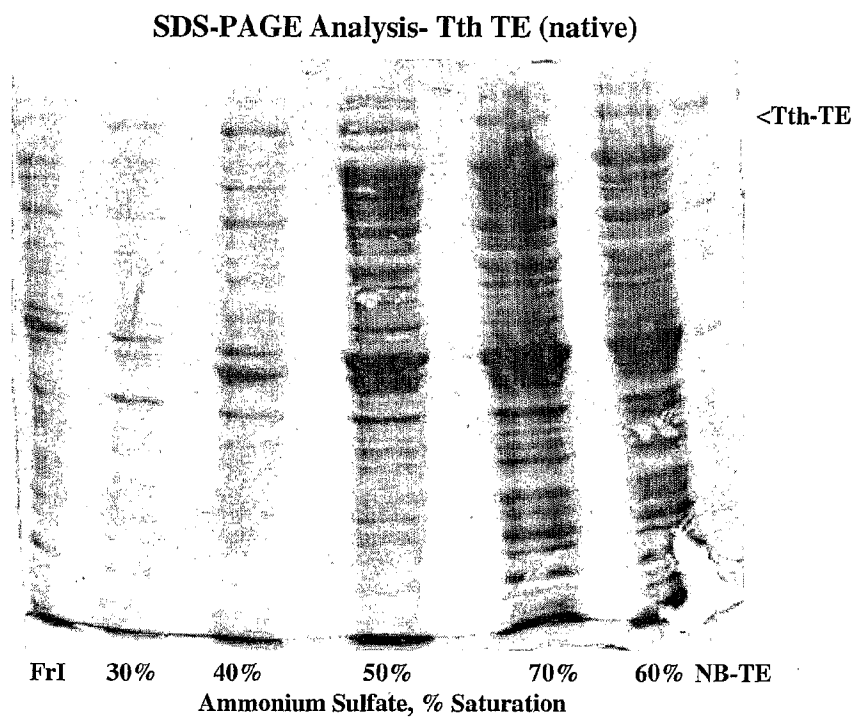


3/63

**FIG. 3**

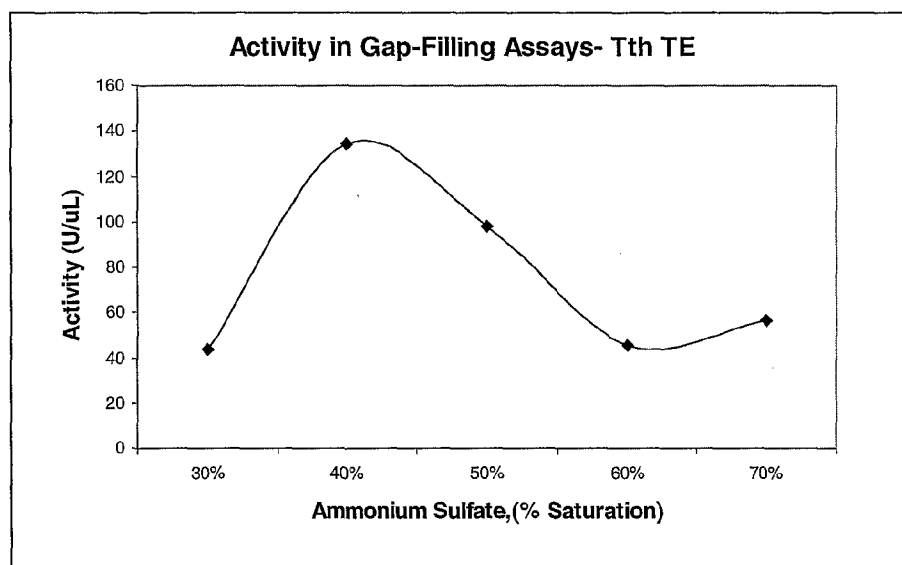
**Protein profile of ammonium sulfate precipitation optimization of  
*T. thermophilus*  $\alpha$ .**

4/63

**FIG. 4**

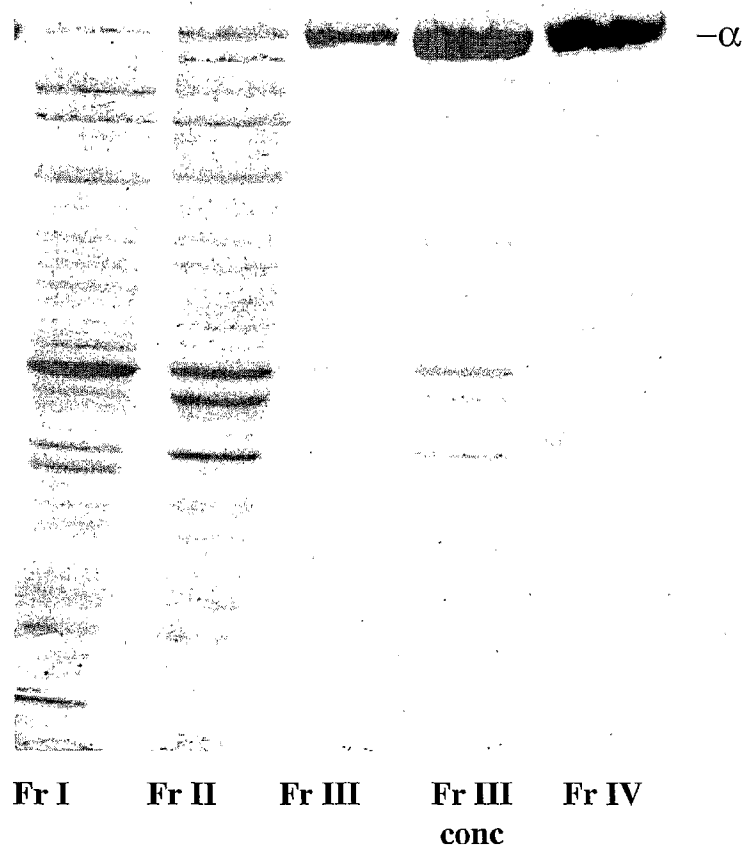
SDS-PAGE analysis of ammonium sulfate precipitation optimization of  
*T. thermophilus*  $\alpha$ .

5/63

**FIG. 5**

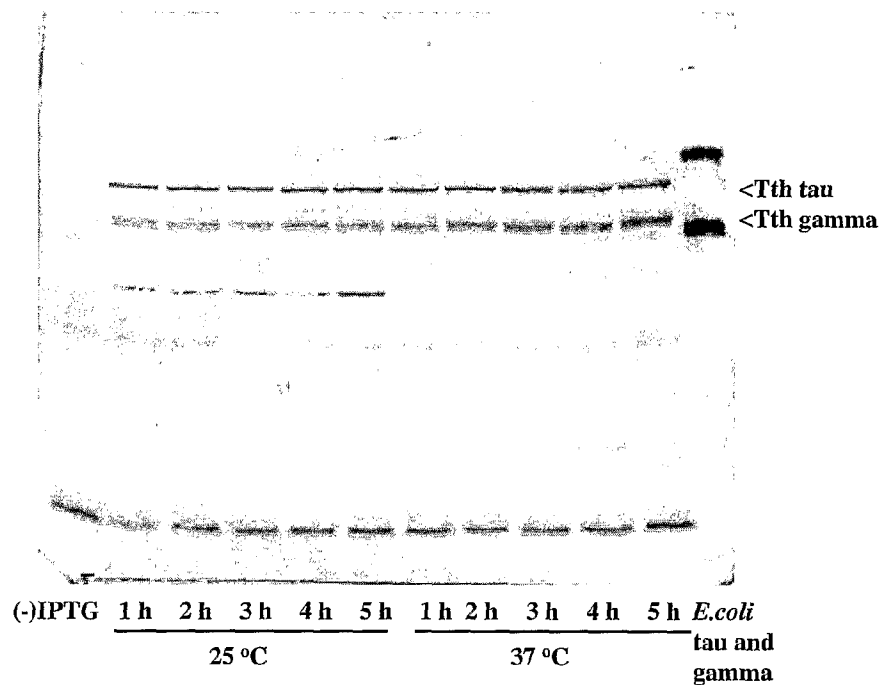
Activity assay analysis of ammonium sulfate precipitation optimization of *T. thermophilus*  $\alpha$  using the gap-filling assay.

6/63



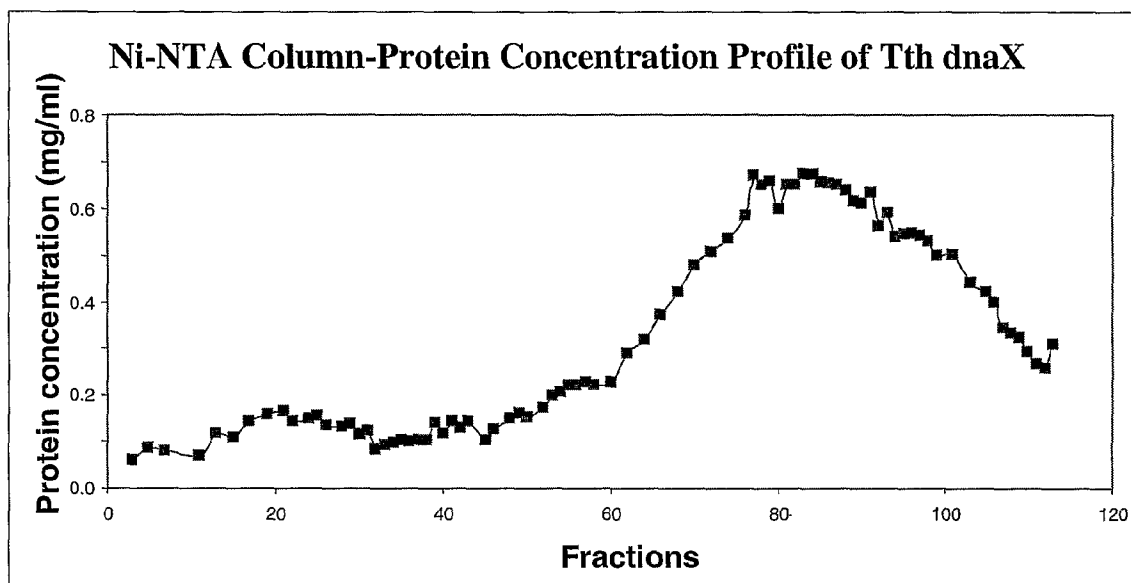
**FIG. 6**  
SDS-polyacrylamide summary gel of the different purification steps of native *T. thermophilus* expressed as a translationally coupled protein.

7/63

**Biotin Blot Analysis- Expression Optimization of Tth *dnaX*****FIG. 7**

**Biotin blot analysis of the growth optimization for expression of N-terminal tagged *T. thermophilus* DnaX subunits from pA1-NB-TX/AP1.L1.**

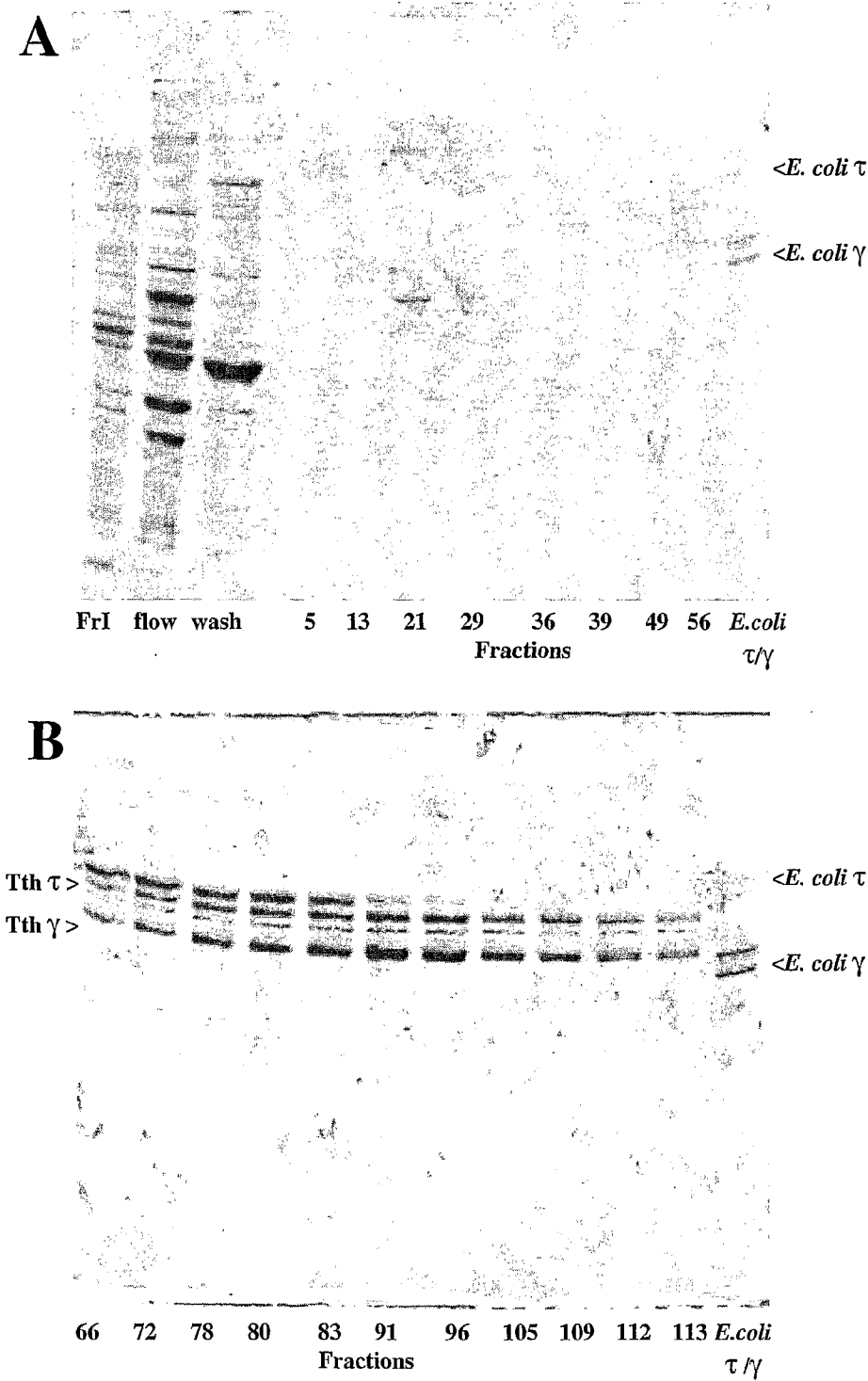
8/63

**FIG. 8**

**Protein concentration profile of the fractions from the Ni<sup>++</sup>-NTA column purification of N-terminal tagged *T. thermophilus* DnaX.**

9/63

SDS-PAGE Analysis- Ni<sup>++</sup> Column Profile of Tth *dnaX*



**FIG. 9A-B**  
SDS-PAGE analysis of the fraction from the Ni<sup>++</sup>-NTA column purification of N-terminal tagged *T. thermophilus* DnaX.

10/63

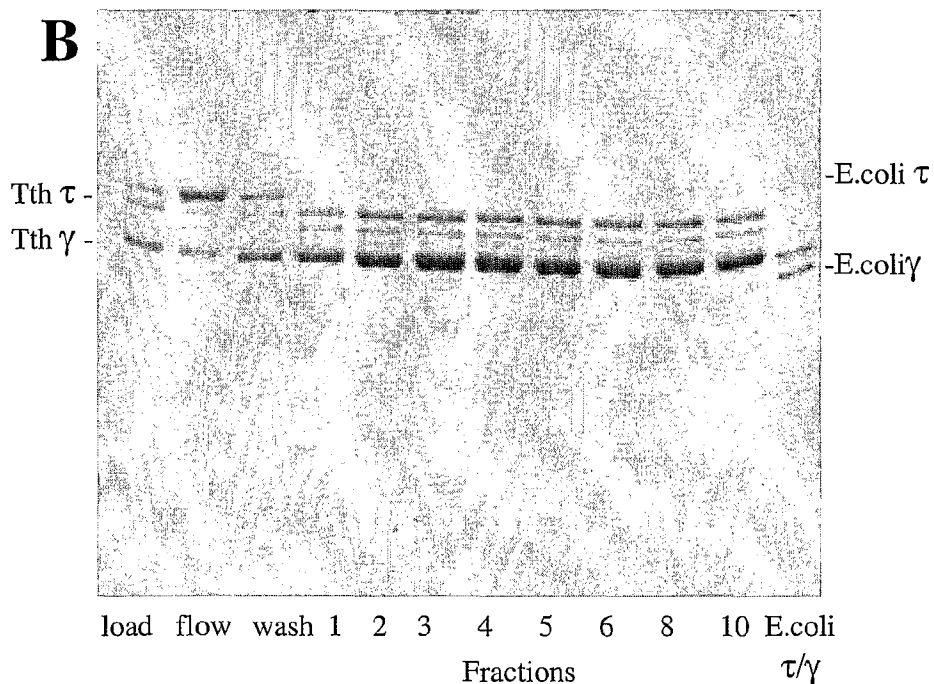
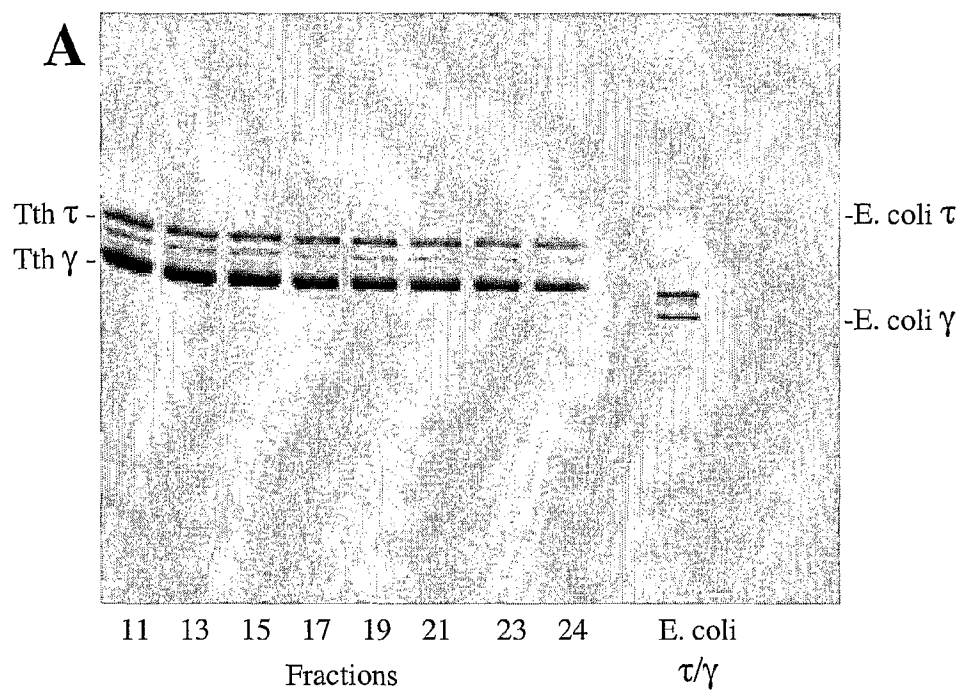


FIG. 10A-B

SDS-PAGE analysis of the fraction from the avidin column purification of  
N-terminal tagged *T. thermophilus* DnaX.



11/63

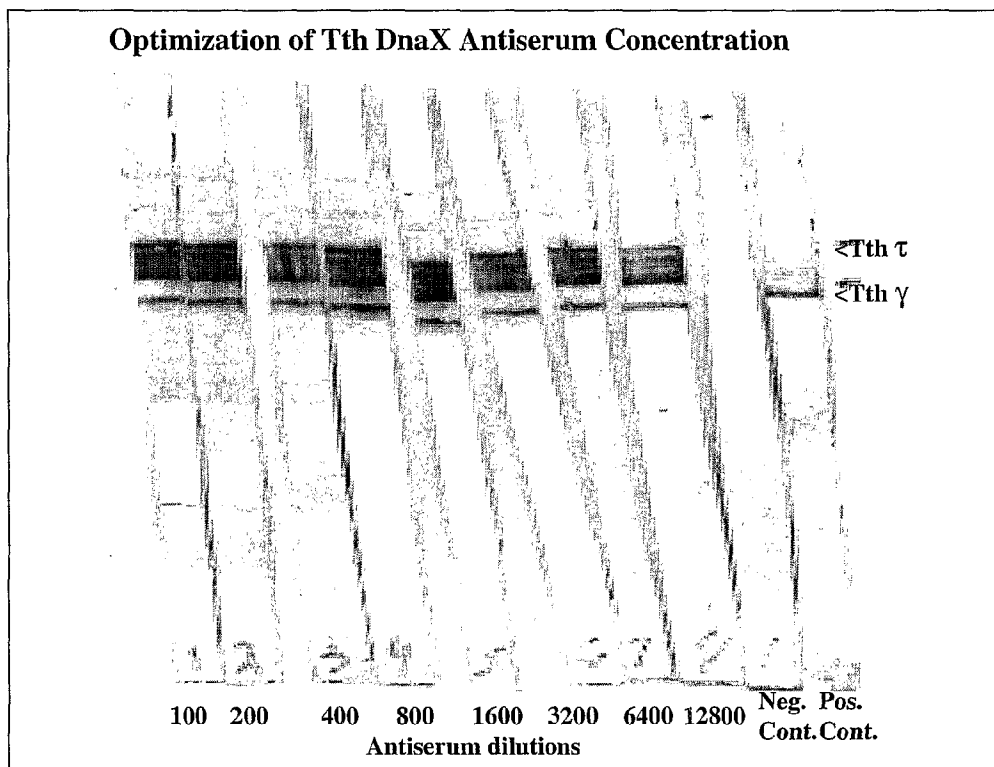


FIG. 11

Western analysis of various antiserum dilutions for determination of dilutions to use in *T. thermophilus* DnaX detection.

12/63

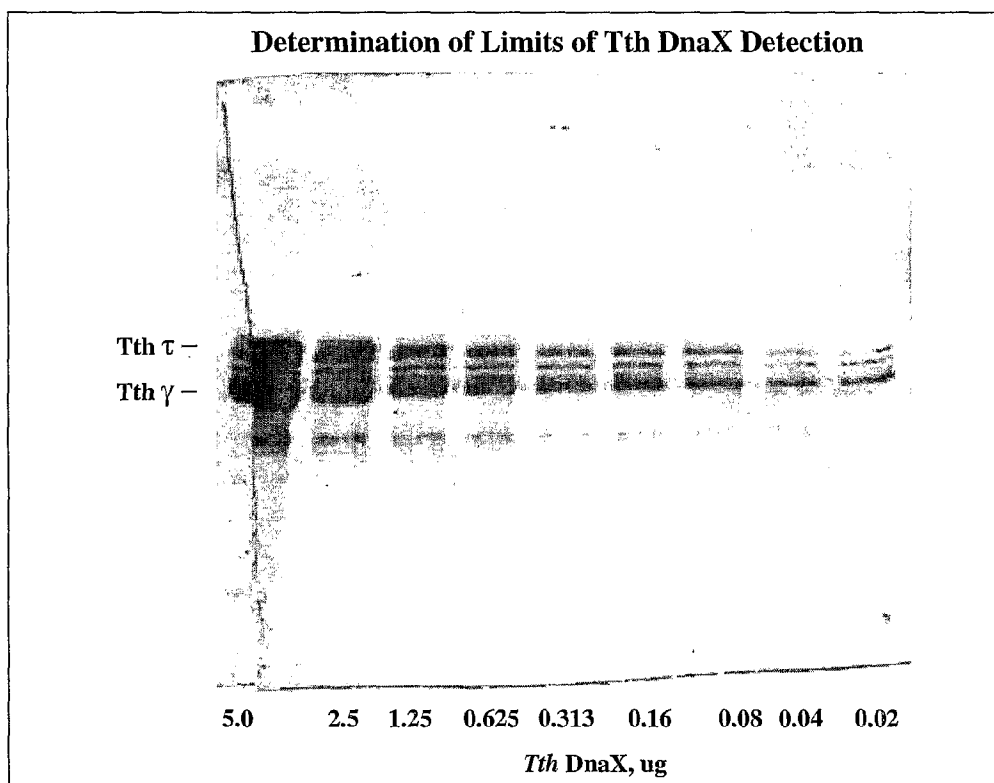


FIG. 12

Western analysis of various *T. thermophilus* DnaX dilutions for determination of the limit of DnaX detection at antiserum dilution of 1:6400.

13/63

**SEQ ID NO:9**

**at**gggtcatcgccttcaccggggatcccttcctggcgcgggagggccctcttagaggaggca  
aggcttagggggcctttcccgccttcaccgagcccaccccgaggccctggcccaggccctc  
gccccggggccttttcggggggcggggggcgatgctggacctgagggaggtgggggagggcg  
gagtgggaaggccctaaagccccctcctggaaagcgtgcccgagggcgctccccgtcctcctc  
ctggaccctaagccaagccccctcccgggcggccttctaccggaaccgggaaaggcgggac  
ttccccaccccccaagggaaggacctgggtgcggcacctggaaaaccgggccaagcgcctg  
gggctcaggctcccgggcgggggtggcccagtaacctggcctccctggagggggacctcgag  
gccttggaaacgggagctggagaagcttgccctcctctccccctcccctcacctggagaag  
gtggagaaggtgggtggccctgagggccccccctcacgggctttgacctgggtgcgctccgtc  
ctggagaaggacccaaggaggccctcctgcgcctcaggcgcctcaaggaggagggggag  
gagccctcaggctcctcggggccctcctcctggcagttcgccctcctcgcccgggccttc  
ttcctcctccgggaaaaccccaggcccaaggaggaggacctcgcccgctcagggccac  
ccctacgccgcctcaaaaaggccctggaggcggcgaggcgccttacggaagaagccctcaag  
gaggccctggacgcctcatggaggcggaaaagaggggccaagggggggaaagacccatgg  
cttgccctggaggcggcggtcctccgcctcgcccgct**tga**

**FIG. 13**

**The DNA coding sequence of the *T. thermophilus* *holA* gene (SEQ ID NO:9).**

14/63

SEQ ID NO:10

MVIAFTGDPFLAREALLEEARLRGLSRFTEPTPEALAQALAPGLFGGGGAMLDLREVGEA  
EWKALKPLLESVPFVLLLDPKPSPSRAAFYRNRRRDFPTPKGKDLVRHLENRAKRL  
GLRLPGGVAQYLASLEGDLEALERELEKLALLSPPLTLEKVEKVVALRPPLTGFDLVRV  
LEKDPKEALLRLRLKEEGEPLRLLGALSWQFALLARAFFLLRENPRPKEEDLARLEAH  
PYAAKKALEAARRLTEEALKEALDALMEAEKRAKGGKDPWLALAAVLRRLAR

**FIG. 14**

The amino acid sequence of *T. thermophilus*  $\delta$ -subunit.

15/63

		1		50
Tthdelta	(1)	-----MVLAF <sup>1</sup> TG <sup>2</sup> DP <sup>3</sup> FLARE <sup>4</sup> ALLEEAR <sup>5</sup> LRGLSR <sup>6</sup> ET <sup>7</sup> EP		
E. coli delta	(1)	MIRLYPEQLRAQLNEGLRAAYLL <sup>8</sup> GN <sup>9</sup> DPL <sup>10</sup> LQ <sup>11</sup> ESQDAVRQVAA <sup>12</sup> QGF <sup>13</sup> EEH		
Consensus	(1)	I DP L EA L A F E		
		51		100
Tthdelta	(32)	TPEALA-----Q--A---LAPGL <sup>51</sup> EGGGAM <sup>52</sup> DIR <sup>53</sup> EVC--EAEWKA <sup>54</sup> KPT		
E. coli delta	(51)	HTFSIDPNTDWN <sup>55</sup> AFSLCO <sup>56</sup> MSL <sup>57</sup> ASRQT <sup>58</sup> LLLPENG <sup>59</sup> NA <sup>60</sup> INEQ <sup>61</sup> LT <sup>62</sup> L		
Consensus	(51)	AI N A LFA LL L E G A L L		
		101		150
Tthdelta	(69)	LESVPEGVPVLL <sup>101</sup> LDEK <sup>102</sup> PSPS--RAAFY <sup>103</sup> RNRERR----DFPTE <sup>104</sup> KGKD <sup>105</sup> VRH		
E. coli delta	(101)	TGLLHDDLLLTIVRG <sup>106</sup> NKLSKAQENAAW <sup>107</sup> TALAN <sup>108</sup> RSVQVTCQ <sup>109</sup> TEQAQ <sup>110</sup> LP <sup>111</sup> W		
Consensus	(101)	L D L LIL K S A AAF R TP L R		
		151		200
Tthdelta	(113)	LENRA <sup>113</sup> RRLGL <sup>114</sup> PLGGVAQ <sup>115</sup> YLAS-LEGDL <sup>116</sup> EALERELE <sup>117</sup> KLALLSP--PLT <sup>118</sup> TE		
E. coli delta	(151)	VAA <sup>119</sup> RAKQLN <sup>120</sup> IEDDA <sup>121</sup> ANQVLCYCE <sup>122</sup> GNLLAQA <sup>123</sup> LERLSL <sup>124</sup> WPDGK <sup>125</sup> LT <sup>126</sup> LP		
Consensus	(151)	L RAK L L L A Q L EG L AL LEKLALL P LTL		
		201		250
Tthdelta	(160)	KVEKV <sup>160</sup> VALRPPL <sup>161</sup> IGFDL <sup>162</sup> VRSVLEKDPKE <sup>163</sup> ALLRI <sup>164</sup> RR <sup>165</sup> LKE <sup>166</sup> CE <sup>167</sup> EP <sup>168</sup> LRL <sup>169</sup> LGA <sup>170</sup> L		
E. coli delta	(201)	RVEQAV <sup>171</sup> NDAAHF <sup>172</sup> TPH <sup>173</sup> WVD <sup>174</sup> ALMGKSK <sup>175</sup> RALHIL <sup>176</sup> QQLR <sup>177</sup> LEGSE <sup>178</sup> PVILL <sup>179</sup> RTL		
Consensus	(201)	KVE V T F V ALL K AL L LK EG EPL LL L		
		251		300
Tthdelta	(210)	SWQFALLARAFFLLRENER <sup>210</sup> PKED <sup>211</sup> LARLEAHPYA <sup>212</sup> AKKAL <sup>213</sup> EAAR <sup>214</sup> LITEAL		
E. coli delta	(251)	QRELL <sup>215</sup> LVNLK <sup>216</sup> QSAHT <sup>217</sup> PLRAL <sup>218</sup> FDK <sup>219</sup> HRVWQN--RRGMMGE <sup>220</sup> ALN <sup>221</sup> RLSQT <sup>222</sup> QL		
Consensus	(251)	LL P D RL EA RLS L		
		301		345
Tthdelta	(260)	KEA <sup>260</sup> LDALMEAEK <sup>261</sup> RAK--GKDP <sup>262</sup> WLAL <sup>263</sup> EA <sup>264</sup> AV <sup>265</sup> RLAR-----		
E. coli delta	(299)	ROAVQL <sup>266</sup> TRTEL <sup>267</sup> TLKDYC <sup>268</sup> QSVWAE <sup>269</sup> LEGLS <sup>270</sup> LLCHKPLAD <sup>271</sup> V <sup>272</sup> FIDG		
Consensus	(301)	K AL L E K G W LEA L L		

FIG. 15

Alignment of the amino acid sequence of  $\delta$  from *T. thermophilus* and *E. coli*.

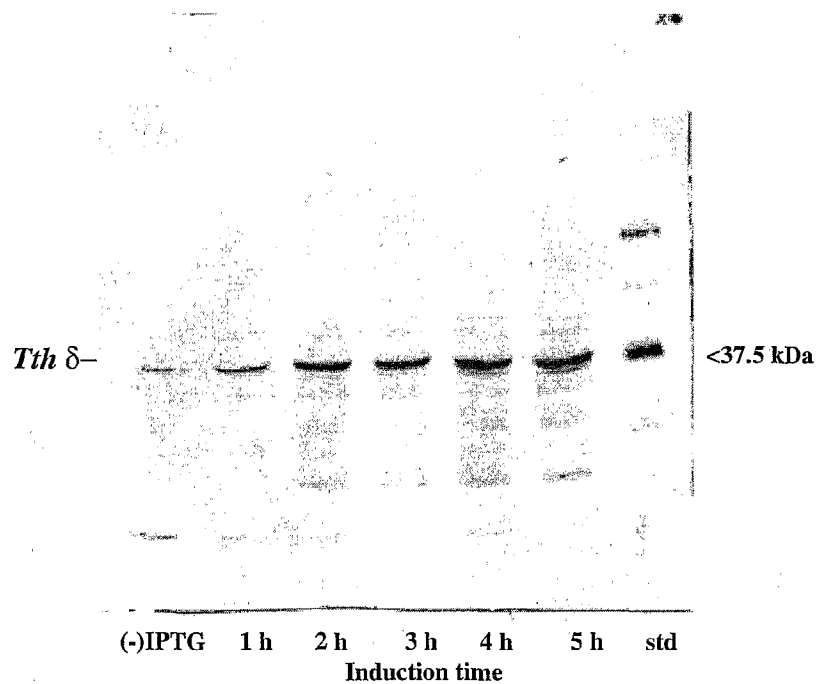
16/63

	1	50
Aquifex delta	(1) METTIFQFQKTFFTKPPKERVVTHCEHOYLIRTFLSKLKEKYGENYTV	
Tthdelta	(1) -----MVIAFTG-DEFLAREALLEEARLRGLSRFTF	
B. subtilis delta	(1) ---MVFQVWVSLKKGE-VHPVYCYGKETYLLQETVSRQRTVVDQETKD	
E. coli delta	(1) -MIRLYPEQLRAQLNEGLRAAYLLCNDLILLQESQDAVROVAAAQGFEE	
H. influenzae delta	(1) -MNRIFPEQLNHHLAQGLARVYLQCODELLLSETEDTICQVANLQGFDE	
Consensus	(1) IF Q L VYLL G DPYLL ET I QV Q F E	
	51	100
Aquifex delta	(50) LWGDEISEEEFYTALSET----SIFGCSKEKAVVIYNFGDFLKKLGRKKK	
Tthdelta	(31) P-----TPEALACALAP-----GLTGGGG-----AMLDLREVG---EA	
B. subtilis delta	(47) FNLSVFDLEEDPLDQAIADAETFPFMCERR--LVIVKNPYFLTGEKKKEK	
E. coli delta	(50) HHTFSTDPNTDWNALFSLCQAMSLFASRQ-----TLLLLPENGPNAA	
H. influenzae delta	(50) KNTIQVDSQTDWAQILIESCQSIGLFFSKQ-----TISLNLFPEN-FTAL	
Consensus	(51) ID E DW ALA LFGG L L	
	101	150
Aquifex delta	(96) EKERLIKVLNRVKS--NYVETVYDAKLQKQELSSEPLKSVAS--FGGIVV	
Tthdelta	(61) EWKALKPTLESVPE--GVPVLLLD---PKPSPSRAAFVRNRE-----RR	
B. subtilis delta	(95) IEHNVSALESYIQSPAPYTVFLLAPYEKLDERKKLTAKKK--HAFMM	
E. coli delta	(93) INEQILLTGTGLLD--DILLIVRGNKLSKAQENAAWETALAN--RSVQV	
H. influenzae delta	(92) IOKNLOELLESVHK--DVLLILOVAKLAKGIEKQTWETTLNQYEPNTILL	
Consensus	(101) I NL LL L VLLIV AKL K E F AL ILV	
	151	200
Aquifex delta	(142) ANRLSK-ERTKQVLKFKFEKCTWENDALEYLLQLTGYNLMELKIEVEK	
Tthdelta	(100) DFPTPKGKDVRHLENRAKRLGLRLPGGVAQYLA-SLEGDLLEALERELEK	
B. subtilis delta	(142) EAKELNAKETTDETVNLAKTEQKTGTBAEHLVLLVNGHLSSTFOETQK	
E. coli delta	(138) TCQTPEQAQPRVVAARAKQINLELDDAANOVLCCYCEGNLLAIAQALER	
H. influenzae delta	(140) NCQTPTVENLPRVVKNRKAMGLDADNEALQOLCYSYENNLALKQALQL	
Consensus	(151) TP L RWV NRAK LGL LD EA QYL EGNLLAL QELEK	
	201	250
Aquifex delta	(191) LIDYASEKKILTDEVKRVAFSVSENVNVHEFVDLLLEKDYEKALKVLD	
Tthdelta	(149) LALLSE---PITLEKVEKVVA-LRPPLTGHDLVRSVLEKDPKEALLRLRR	
B. subtilis delta	(192) LCTFIGDREELTDVVKMLVA-RSLEQNLIELINKLVNRKRTESLOIFYD	
E. coli delta	(188) LSLWPDG-KLTLPRVEQAVN-DAAHFTPEHWDALLMCKSKRALHTLQQ	
H. influenzae delta	(190) LDLLYPDH-KLNYNRVISVVE-QSSIFTFQWIDALLVGKANRAKRLKG	
Consensus	(201) L LL PD LTLDRV VV S T FEWVDALLKK RAL IL	
	251	300
Aquifex delta	(241) LISFGIHPLOTMKILSSYALKLYTLKLE-----EKGEDLNKA---MESV	
Tthdelta	(195) LKEEGEEPRLGALSQWQFALLARAFFLLR---ENPREKEED---LARR	
B. subtilis delta	(241) LLKONEEPKIMALTISNQFRLLQTKYFA-----EQYGQKQT---ASNL	
E. coli delta	(236) LRLEGSEPVILLRTILORELLLVNLR-Q-----SAHTPLRAI---FDKH	
H. influenzae delta	(238) LQAEQVQVILLRTILORELLTLETKPQQRIVTTEKLELQOIKTEFDRL	
Consensus	(251) L EG BPL LLR LS LLL LKR E PL I DRL	
	301	350
Aquifex delta	(283) GIKNNFLKMKFKSYLKANSKEDLNLLSTQRIDAFSKLYFQDTVOLLRR	
Tthdelta	(238) EAHPYAAKKALEAARRI--TEBAKKEALDAIMEAEKRAKG--GKDPWLALE	
B. subtilis delta	(283) KVHPFRVKLAMDQARLF-SEEEELRLITEQLAVMDYEMTG-KKDKQLLLE	
E. coli delta	(277) RYWNRRRGMMGRALNRL-SQTOLRQAVQLITRIELTLKQDYQGSVWAELE	
H. influenzae delta	(288) KIWONRRPLFLSAIQRL-TYQTLVETIQELANIERLAKQEFSDDEVWIKLA	
Consensus	(301) KI QNR KL LEA RL S E LK II L IE AK F DVWL LE	
	351	368
Aquifex delta	(333) FLTSRLEREVVKNITSHGG	
Tthdelta	(285) AAVLRIRAR-----	
B. subtilis delta	(331) LFLILQLLKRNEKNDPHY-	
E. coli delta	(326) GLSLLLCHKPLADVFIDG	
H. influenzae delta	(337) DLSVKICL-----	
Consensus	(351) LSLRL R	

FIG. 16

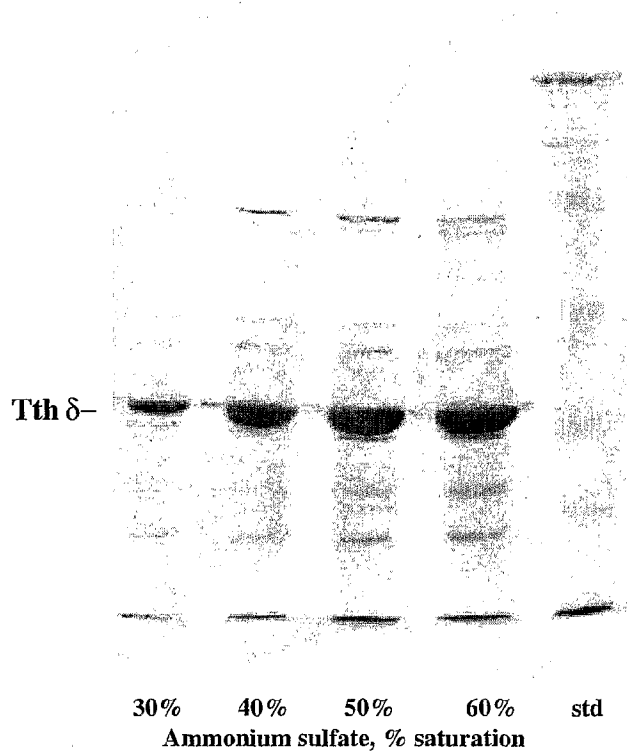
Alignment of the amino acid sequence of  $\delta$ -subunit from *A. aerolicus*,*T. thermophilus*, *B. subtilis*, *E. coli* and *H. influenzae*.

17/63

**Biotin Blot-Expression Optimization of pA1-NB-TD/AP1.L1****FIG. 17**

**Biotin blot analysis of growth/induction time optimization of expression of *T. thermophilus*  $\delta$  by pA1-NB-TD/AP1.L1.**

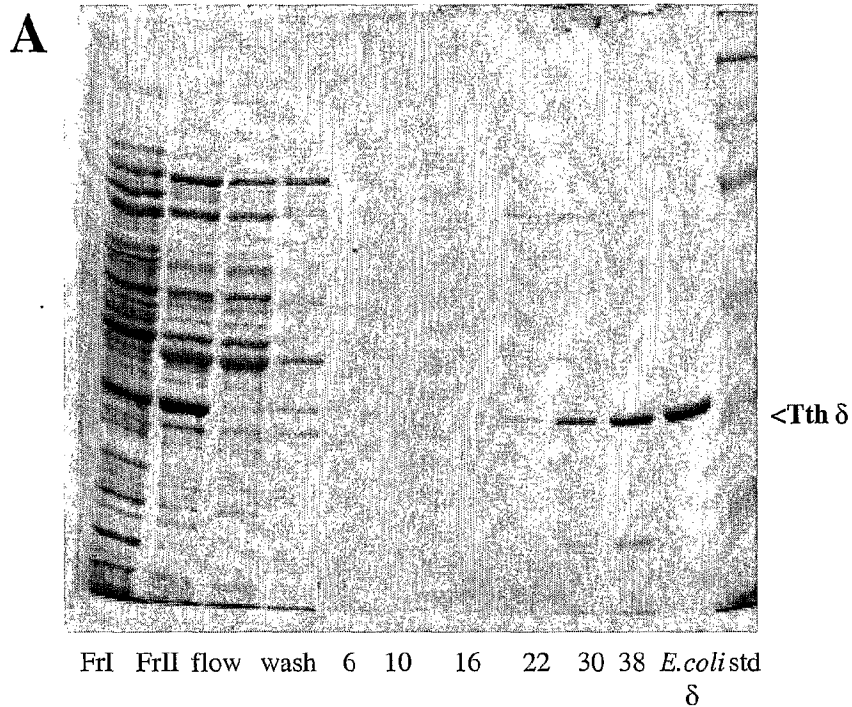
18/63

**SDS-PAGE Analysis-AS Optimization of *Tth*  $\delta$** **FIG. 18**

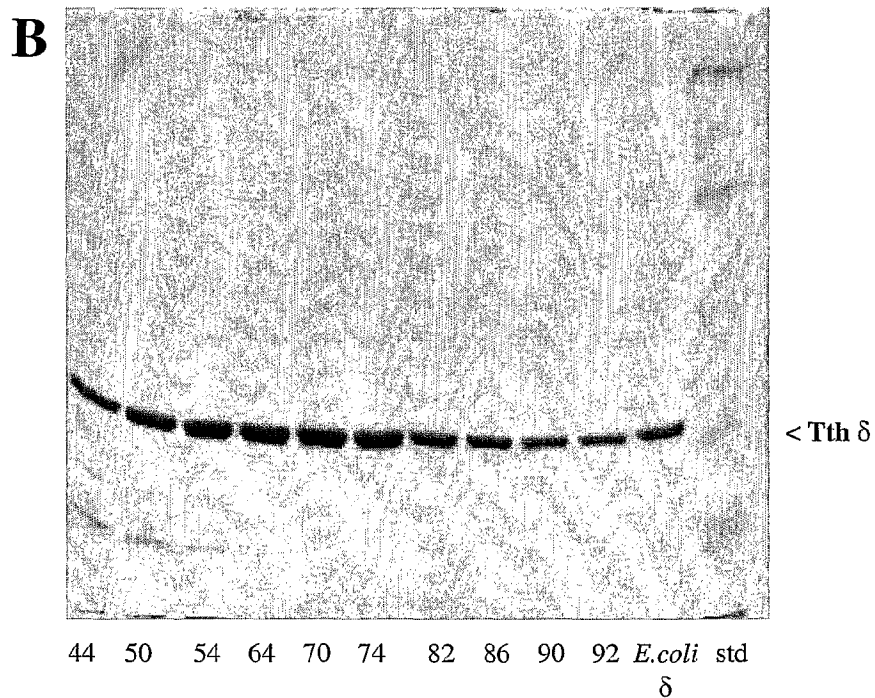
Optimization of precipitation of *T. thermophilus*  $\delta$  by ammonium sulfate.



19/63

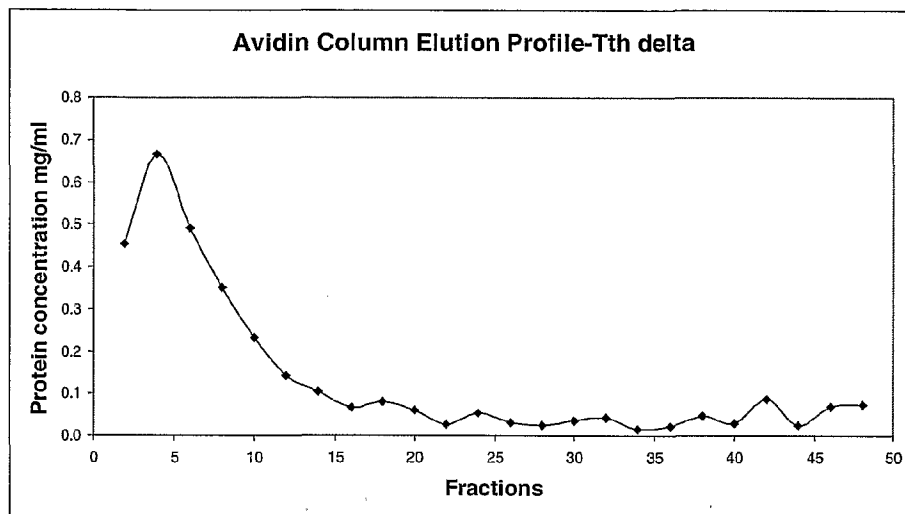
SDS-PAGE Analysis of Ni-NTA Column Profile-Tth  $\delta$ 

## SDS-PAGE Analysis of Ni-NTA Column Profile-Tth delta



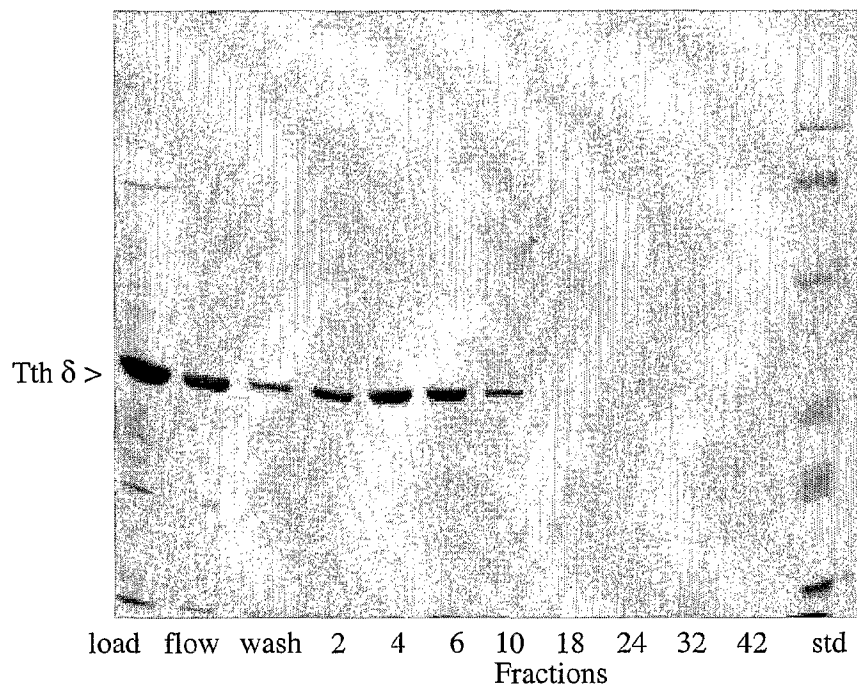
**FIG. 19A-B**  
 SDS-PAGE analysis of fractions from the Ni<sup>++</sup>-NTA column purification of *T. thermophilus*  $\delta$ .

20/63



**FIG. 20**  
Protein concentration profile of fractions from the avidin column  
purification of *T. thermophilus*  $\delta$ .

21/63

**SDS-PAGE Analysis of Avidin Column Profile-Tth  $\delta$** **FIGs. 21**

**SDS-PAGE analysis of fractions from the avidin column purification of *T. thermophilus*  $\delta$ .**

22/63

## SEQ ID NO:16

atggctctacacccggctcaccctggggcaataatcgggcacgaggccgttctcgccctc  
cttccccgcctcaccgcccagaccctgctcttctccggccccgagggggtggggcggcgc  
accgtggccccgctggtaacgcctgggggctcaaccgcggcttccccccgcctccctgggg  
gagcaccgacgtcctcgaggtagggcccaaggcccggaacctccggggccgggcccag  
gtgcggctggaggaggtggcgccctcttgagtggtgctccagccacccccgggagcgg  
gtgaaggtaggcatcctggactcggcccacctcctcaccgaggccgcggccaacgccctc  
ctcaagctcctggaggagcccccttctacgcccgcacgtcctcatcgccccaaagccgc  
gccaccctcctccccaccctggcctcccgggccacggaggtggccttcgcccccgtagcc  
gaggaggccctgcgcgcccttaccaggaacccggggctcctccgctacgcgcggggcc  
ccgggcccgcctccttagggccctccaggacccggaggggtaccgggcccgcacggccagg  
gcgcaaagggtcctgaaagccccgcccctggagcgcctcgccctgcttcgggagcttttg  
gccgaggaggaggggggtccacgccctccacgcgcctcctgaagcgcgggagcacctcctt  
gccctggagcgggcccgggagggccctggaggggtacgtgagccccgagctggtcctcgcc  
cggctggccttagacttagagacatga

## FIG. 22

The DNA sequence of the *T. thermophilus* *holB* gene encoding the  $\delta'$ -subunit  
of the *T. thermophilus* PolIII holoenzyme.

23/63

**SEQ ID NO:17**

MALHPAHPGAIIGHEAVLALLPRLTAQTLLFSGPEGVGRRTVARWYAWGLNRGFPPPSLG  
EHPDVLEVGPKARDLRGRAEVRLEEVAPLLWCSSHPREVRKVAILDSAHLLTEAAANAL  
LKLLEPPPSYARIVLIAPSRATLLPTLASRATEVAFAPVPEEALRALTQDPGLLRYAAGA  
PGRLLRALQDPEGYRARMARAQRVLKAPPLERLALLRELLAEEEGVHALHAVLKRPEHLL  
ALERAREALEGYVSPELVLARLALDLET

**FIG. 23**

24/63

```

1                               50
E. coli delta prime (1) MRWYPWLRPDFEKIVASYQAGRGHALLTQALFGMGDDALIVALSRYLCLC
Tth delta prime (1) -----MATHPAHPGATIGHEAVLALLERLTAQTLLFSGPEGVGR
Consensus (1) L AH AA H LI LP L LIFA L
51                               100
E. coli delta prime (51) QQPQGHKSCGCHRCGCOLMQACTHPTVYTLAPEK---GKNTLGVDVAVREV
Tth delta prime (40) RTVARWYAWGLNRGFPFPPSLCEHPDVLEVGPKARDLRCRAEVRLEEVAPF
Consensus (51) A G RG G HPD LAP GK L LD V L
101                               150
E. coli delta prime (97) TEKLNHARLGGAKVWVYTDALLTDAANALKLTLEEPPAETWFFLATR
Tth delta prime (90) LEWCSSHPER-VKVAILLDSHLLTEAANALKLEEPPSYARIVLIAP
Consensus (101) E H R KV L A LLTDAANALLK LEEPPA L
151                               200
E. coli delta prime (147) EPERLLATLSRCLHYLAPEPEQYAVTWLSREVTMSODALLAALRLSAG
Tth delta prime (139) SRATLLPTLASRATEVAFAPVPEBALR-----ALTQDP--GLRYAAG
Consensus (151) LL TL SR AP PE LSQD A LR AAG
201                               250
E. coli delta prime (197) SPGAALALFQ-GENWOARETLCQALAYSVPESGDWYSLAALNHEQAPARI
Tth delta prime (180) APGRLLRALQDPEGYRARMARAORMLKAP-----LERI
Consensus (201) APG L Q DW AR Q L A P RL
251                               300
E. coli delta prime (246) HWLATLMDALKRHHGAAQVTNVDVPGLVAEANHLSESRLOAIIGDVCH
Tth delta prime (214) ALLRETLABEEGVHALHVLK-----REE---HLLATER
Consensus (251) L LL D H A L P L L
301                               339
E. coli delta prime (296) IREOLMSVTGINRELLTDLRLTEHYLQPGVVLPVPHL
Tth delta prime (245) AREALEGYVSP--ELVLARALDET-----
Consensus (301) RE L ELLI L L IE

```

FIG. 24

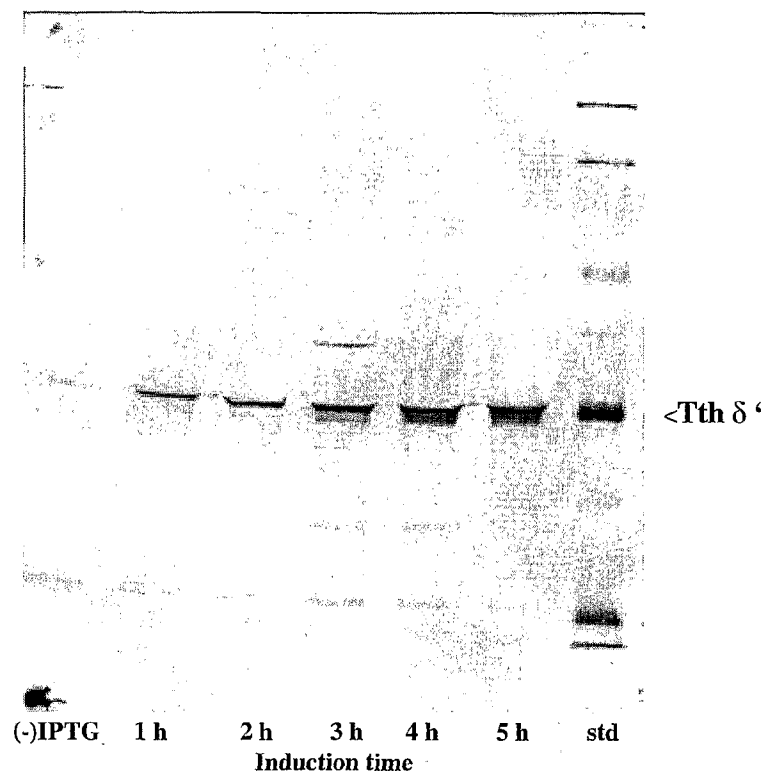
Alignment of the amino acid sequence comparing *E. coli* and *T. thermophilus*  $\delta^+$ .

25/63

	1	50
A. aeolicus delta'	(1) -----MEKVFLERKIQ---KTLHTPCGLIFYGKEGSKTKTAF	
B. subtilillus delta'	(1) MAISWK---EMNETQFRVMKLTLYNSIEKDRLSHAYLFEGKKGTCKLDAAL	
E. coli delta'	(1) MR-----WYPWLRDEDEKIVA-SYQAGRGHALLTQALPGMDDADLY	
H. influenzae delta'	(1) MEIKRMTALYPWMEIYHQTAQ--TFDEGLGHFAVLTKADSGLEVESHFN	
R. prowazekii delta'	(1) -----MTILOELIETLBFYLYKYNKLYNSWLEAWN---IEQTILQ	
Tth delta'	(1) -----MALHP-AHPGATIGHEAVLALPRLTATQTLF	
Consensus	(1) M L FIFM LL S GRHHALLI A GLG E LLF	
	51	100
A. aeolicus delta'	(35) EFARGFLCKE-NVPWGCSCSPCKHVNELEEAFFKGEIEDFKVYKDKDGK	
B. subtilillus delta'	(48) LLAKSFFCLE-GCAPECESCRNCKRI	
E. coli delta'	(43) ALSRYLCCQQPGGHKSCGHCRCQQLM-----	
H. influenzae delta'	(50) ALAOKIMCVA-CGDKECGCHSCHLM-----	
R. prowazekii delta'	(38) DLKEFYIKFFNSNILENNPDYFV-----	
Tth delta'	(32) SGPEGVGRRTVARWYAWGLNRGFPP-----	
Consensus	(51) ALAK ILCK NG PCG CR C M	
	101	150
A. aeolicus delta'	(84) KHPVYLMCEHPDEFVVTIP-----SCHY-TKLEQIREVKNFAYVKPALSR	
B. subtilillus delta'	(73) -----ESGNHPDLHLVOP-----DGLS-IRKKAQIQALQEFKTKGLSHK	
E. coli delta'	(69) -----QAGTHPDYITLAP-----BKCKNTIGVDAVREYTEKINEHARLGG	
H. influenzae delta'	(75) -----QAHSHPDYHRLSP-----INGKD-TGVDOVRDINEMVACHAQQCN	
R. prowazekii delta'	(64) -----ARNDSYTSNA-----KN-TSTEQIRKLODELNKTSAISGY	
Tth delta'	(58) -----SLGEHPDVLVVGKARDLRGRAEVRLBEVAPILNCCSSIPRER-V	
Consensus	(101) AG HPDYH L P GK IKLEQIRELQEFLS HA LSG	
	151	200
A. aeolicus delta'	(128) KVLITDDAHAMISQAAANALLKVLLEPPADTTHLTINRRSAILPTILSR-	
B. subtilillus delta'	(112) KLYITISHADQMTANAANSLLKFLLEPNKOTMAVLITEQQRLLDTTISRC	
E. coli delta'	(110) KVVVWTDALITDAANALLKTLLEPPAETWFLAREPERLLATLISRC	
H. influenzae delta'	(115) KVVYVQGAERITEAAANALLKTLLEPPRENTYELLQADSSISLATTISRC	
R. prowazekii delta'	(98) KVAVIYSADLMNLHAANALCKILEDTKNSYIFLITSRASITITISRC	
Tth delta'	(102) KVAITLDSAHLETEAAANALLKLEPPSYARIVLTAPSRAITLPLELASR-	
Consensus	(151) KVVII ADLLTEAAANALLKILEPPADTYFVLIT ASLLATI SRC	
	201	250
A. aeolicus delta'	(177) -----TFQVEFKGFSVKEVMEIAKVDEETAKLSG--CSLKRATLLKE	
B. subtilillus delta'	(162) QTLPPQPLQPKAIEDRLIEQDVSPHMARLLNMTNNVABAVELSRN-DEF	
E. coli delta'	(160) RLHYLAPPPEQYAVTWLSREVTMSQDALLAATRLSAGSPCAALALFGDN	
H. influenzae delta'	(165) QVWNLSVPNEETAFEWLKKSASVENQETITATAMNLGRPLLAETLQEGF	
R. prowazekii delta'	(148) -----FKVNIRSPLPNVSNIDYLOQIQTADNKTLL-DF	
Tth delta'	(151) -----AT-EVAFAEVPEEALRAITQDPGLRYAAGAPGRLLRALQDPE	
Consensus	(201) VAF L VM L VL AL LNAGAGA LR LQDDF	
	251	300
A. aeolicus delta'	(217) NKDILNKVKEFLENBFLK-----VYKASEFEKWE-PEKQKLFLE	
B. subtilillus delta'	(211) AESRAKVIKLYEVLHQRKGHAFFFIQDQWMPFFKEKTHQ--EMGLDMLLL	
E. coli delta'	(210) WQARETLQALAYSVBSG-----DWYSLLAANNHCOAPARLHWTAT	
H. influenzae delta'	(215) IEQRKNFLRQFWVFYRRR-----SPLELLPLPDKERYVQVQDWLDA	
R. prowazekii delta'	(180) INRFTTKDRELWLGFIEN-----LFLMNRHLKK--SVNFNIDLL	
Tth delta'	(193) GYRARMARAQRVLKAEPL-----ERLALIREHLAE--EEGVHADHA	
Consensus	(251) R IK F L P K Y LL EL KE L ILL	
	301	350
A. aeolicus delta'	(256) IMEELVSQKLTBEKKDNYTYLLDITRLFKD-GLARGVNEPLWLFTLAVQA	
B. subtilillus delta'	(259) TYRIVLSTIQIGNEKLIYQDLFQSTKQHALQSTQQSVTNCHL-AVLEAKK	
E. coli delta'	(251) LLMALKRHHGAAQVTNV-DVPGLVABEIAN-HISPSRLQALGQVCHIRE	
H. influenzae delta'	(256) FESCECKHKEIDSHROVADLGRGTEQFSDEQTAQLGLQATK-IMQKVR	
R. prowazekii delta'	(218) DENKTYNKINKNPAYLL-QKFTDKKLIYNTIDYDLKTS-YTLVYNE	
Tth delta'	(232) VIK-RPEHLALERAREA-LEGVVSPIVLAFLALDIET-----	
Consensus	(301) IL D L KLG E NV DL IK LA LA L Q I VL VR	
	351	387
A. aeolicus delta'	(305) D-----	
B. subtilillus delta'	(308) RHSHN--VNVQGLMEHLVLMLOEG--	
E. coli delta'	(299) QEMSVTGINRELLITDILLR-IEHYLQPGVLPVPHL	
H. influenzae delta'	(305) DDLINGVNVVELMLDGLTRLVTEVFETQ-----	
R. prowazekii delta'	(266) FFTV-----	
Tth delta'	(269) -----	
Consensus	(351) L S VN LI L	

FIG. 25

26/63

**Biotin Blot Analysis- Growth Optimization of *Tth*  $\delta$** **FIG. 26**

**Biotin blot analysis of growth/induction time optimization of expression of *T. thermophilus*  $\delta'$  by pA1-NB-TD'/AP1.L1.**



27/63

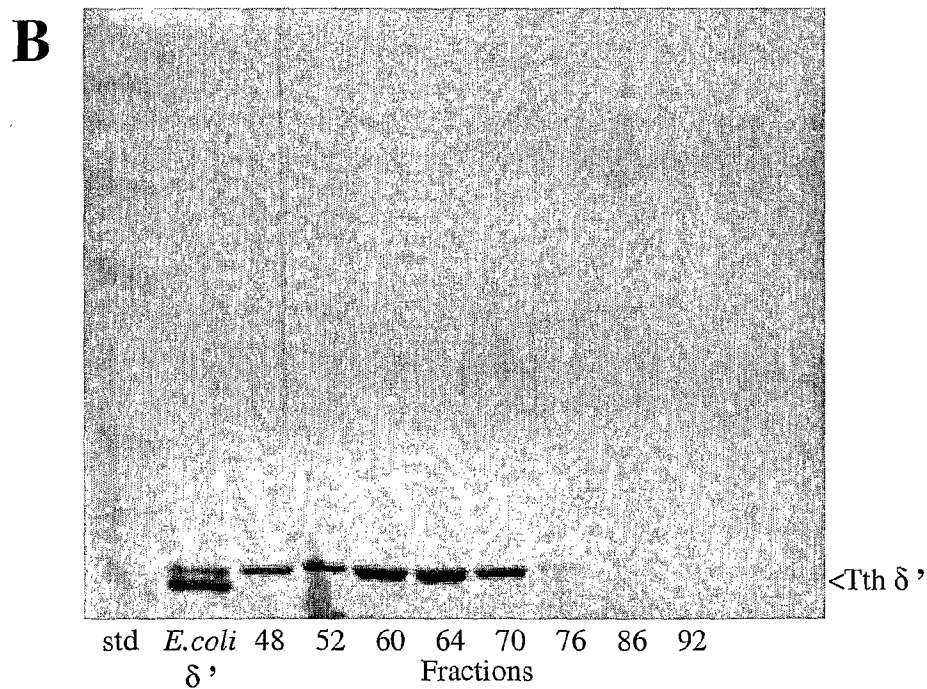
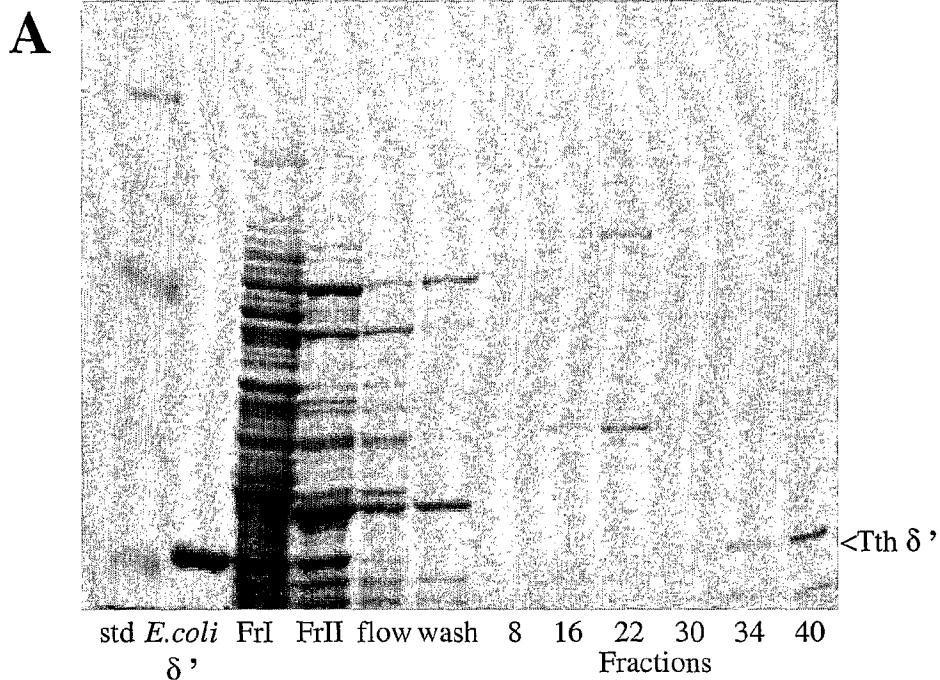
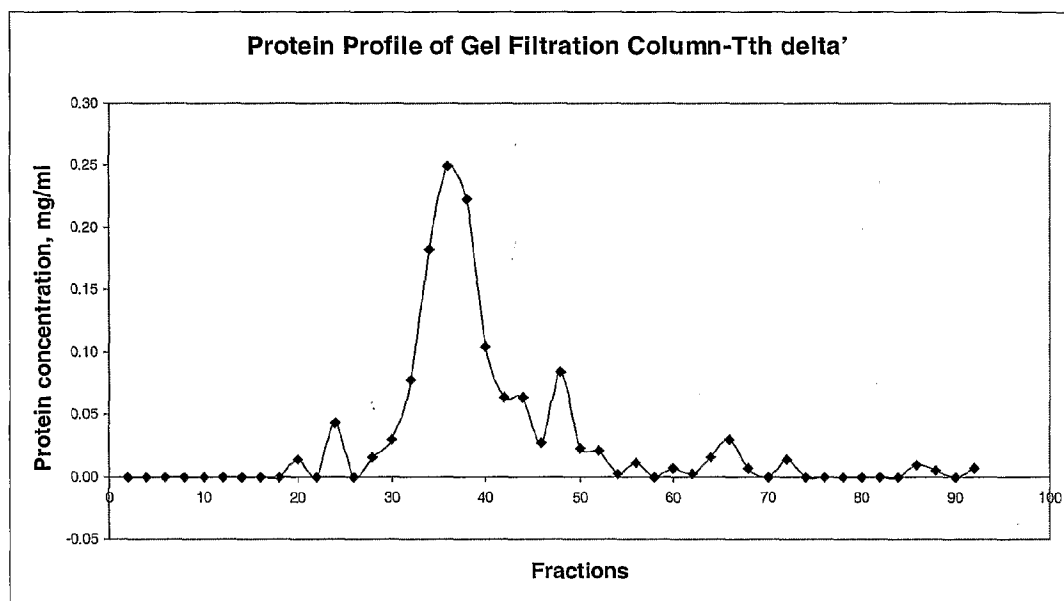
SDS-PAGE Analysis of Ni-NTA Column Profile-Tth  $\delta'$ 

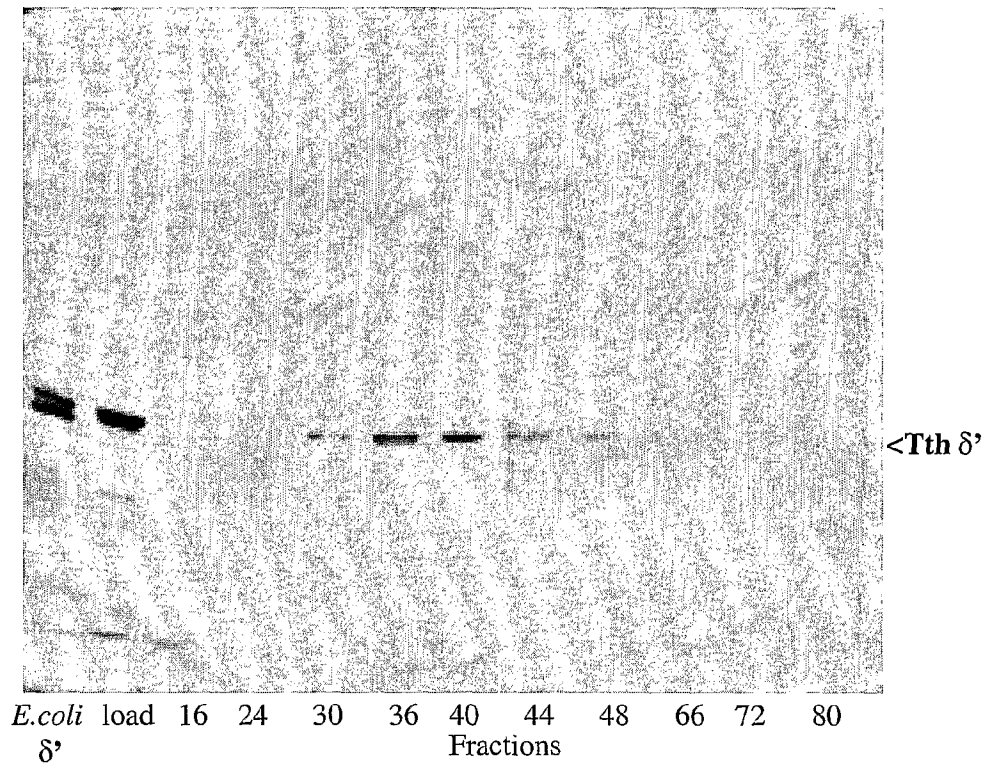
FIG. 27A-B

28/63



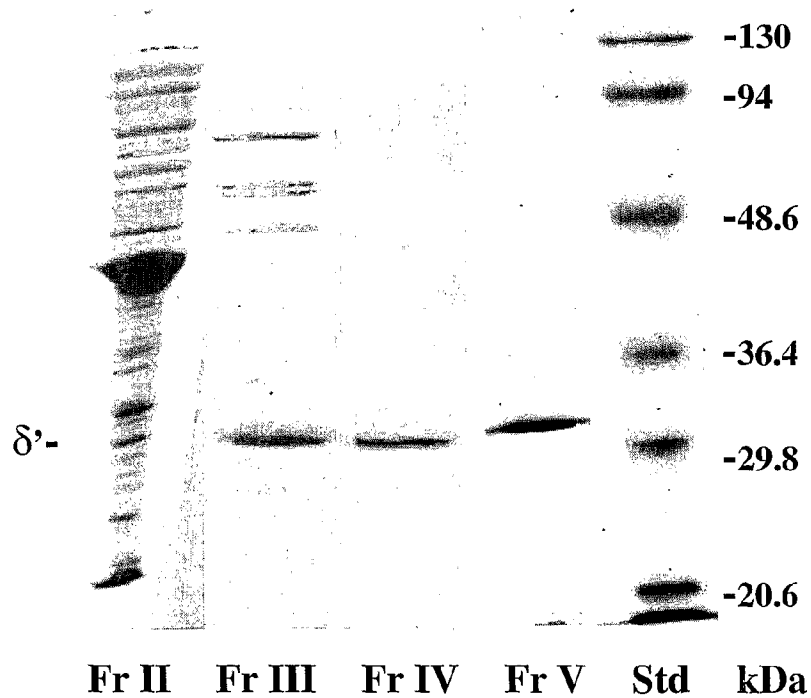
**FIG. 28**  
Protein concentration profile of fractions eluting from the Sephacryl S-300  
gel filtration column purification of *T. thermophilus*  $\delta'$ .

29/63

**SDS-PAGE Analysis of Gel Filtration Column Profile-Tth  $\delta'$** **FIG. 29**

**SDS-PAGE analysis of fractions from the Sephacryl S-300 column purification of *T. thermophilus*  $\delta'$ .**

30/63



**FIG. 30**  
SDS-PAGE summary of the purification of *T. thermophilus*  $\delta'$  as a translationally coupled protein.

Biotin Blot Analysis-Expression Optimization of *Tth* β

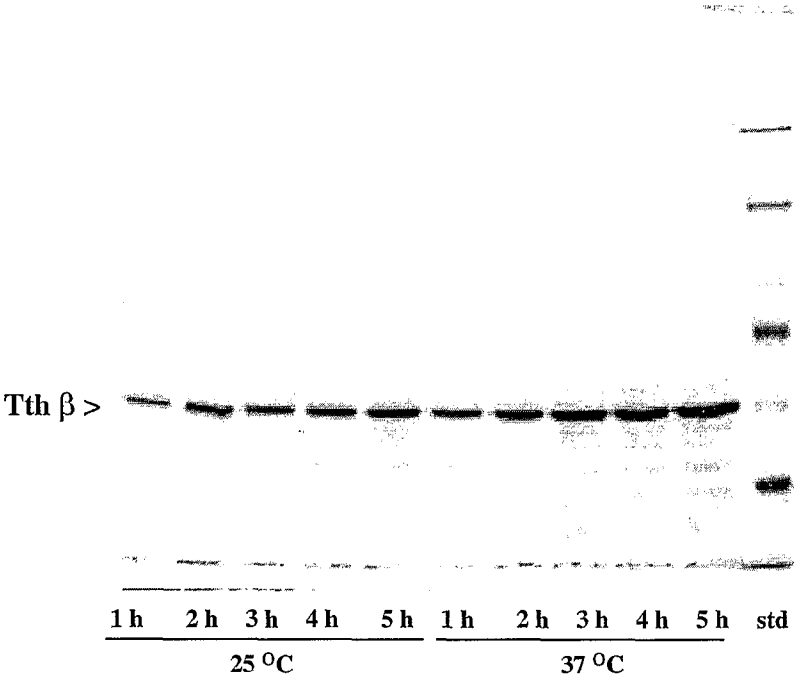
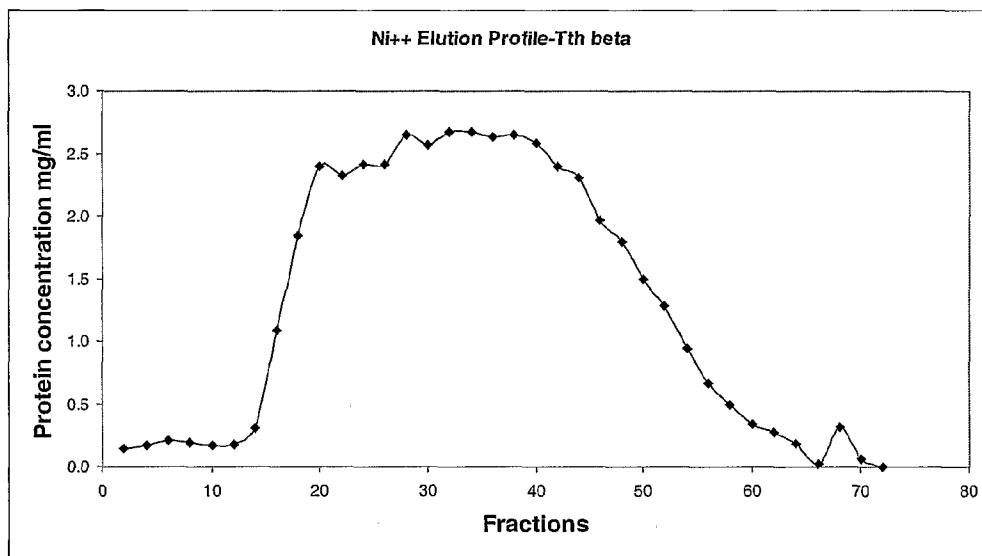


FIG. 31

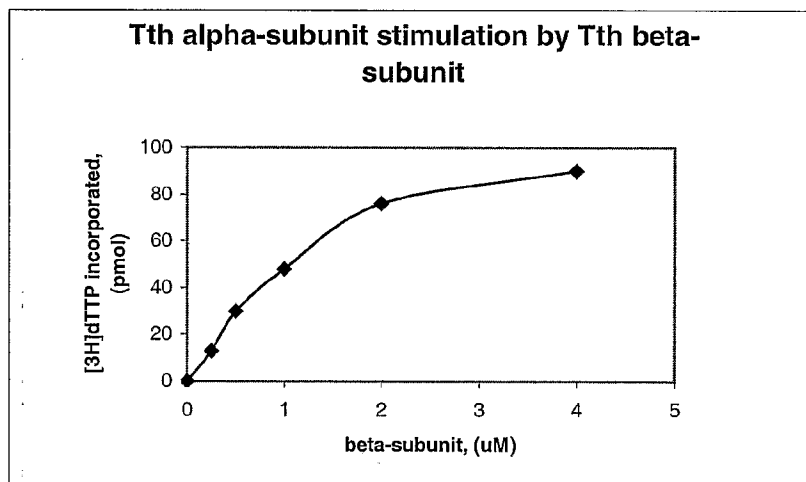
Biotin blot analysis of growth/induction time optimization at different temperatures of expression of *T. thermophilus* β by pA1-NB-TN/AP1.L1.

32/63



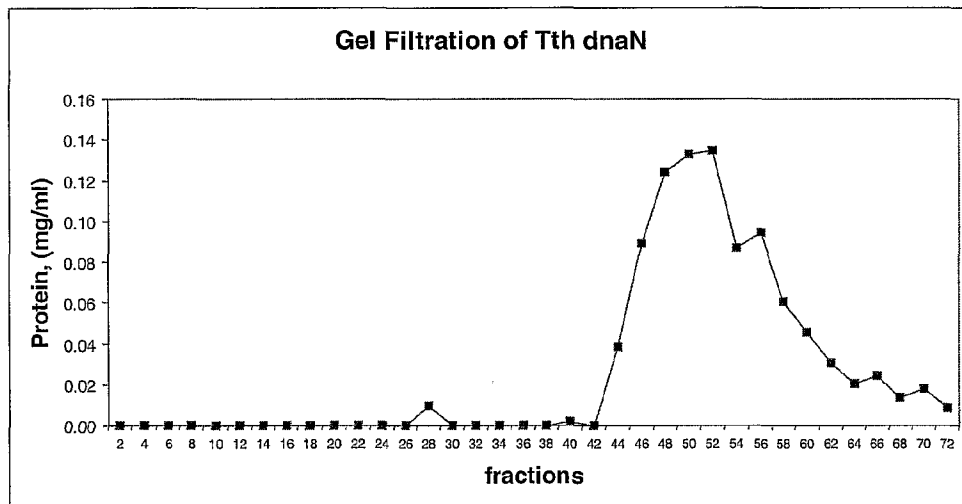
**FIG. 32**  
**Protein concentration profile of fractions eluting from the Ni<sup>++</sup>-NTA column  
purification of *T. thermophilus* β.**

33/63

**FIG. 33**

Primer extension assay to determine stimulation of *T. thermophilus*  $\alpha$  by *T. thermophilus*  $\beta$ .

34/63

**FIG. 34**

**Protein concentration profile of fractions eluting from a Sephacryl S-300 gel filtration column purification of *T. thermophilus*  $\beta$ .**



35/63

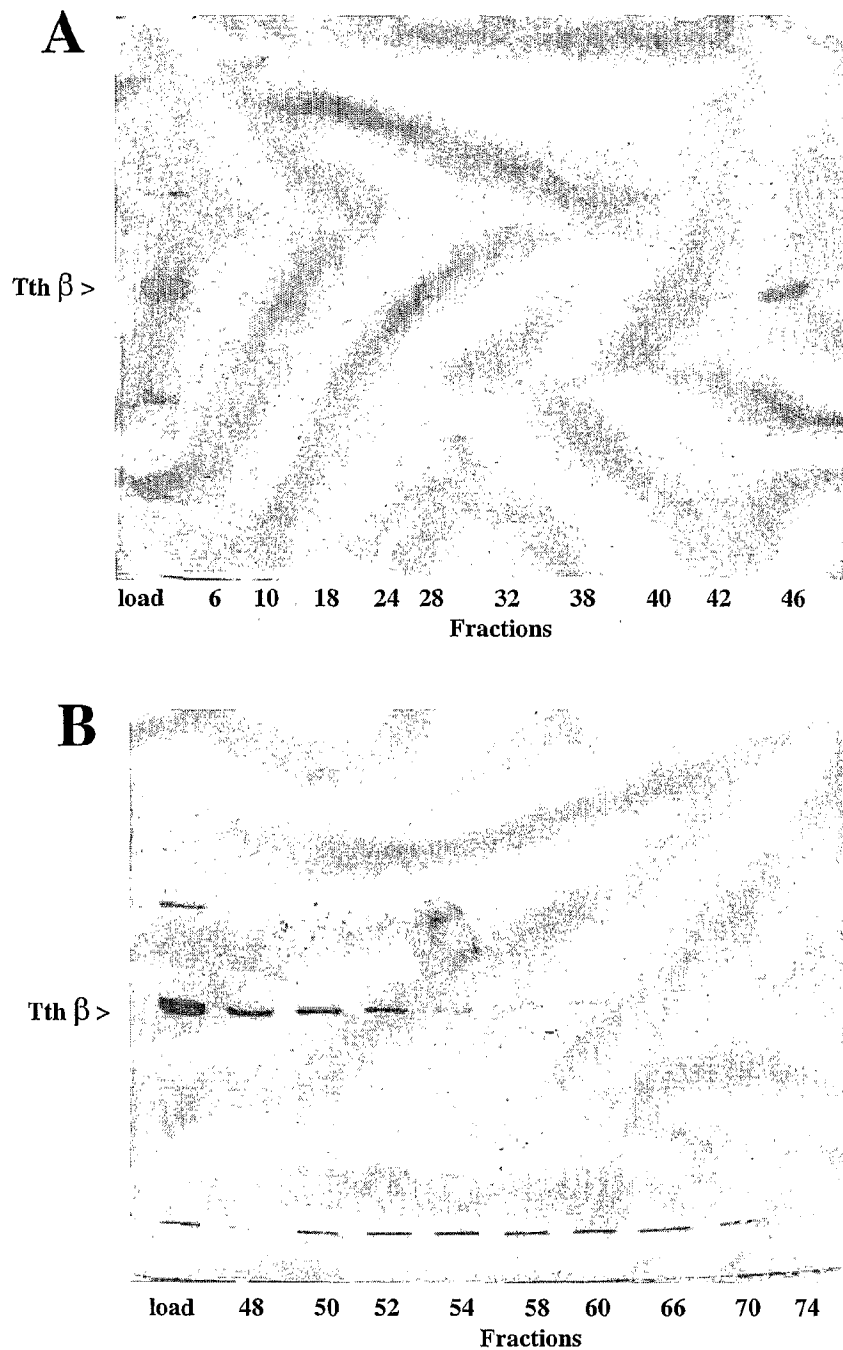
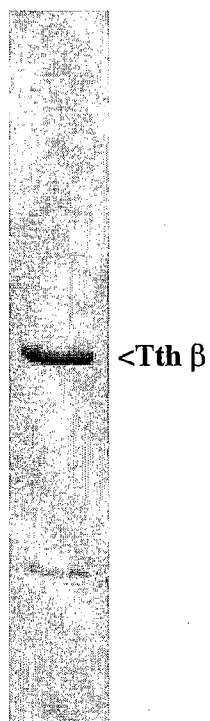
SDS-PAGE Analysis-Sephacryl S-300 Column of *Tth*  $\beta$ 

FIG. 35A-B

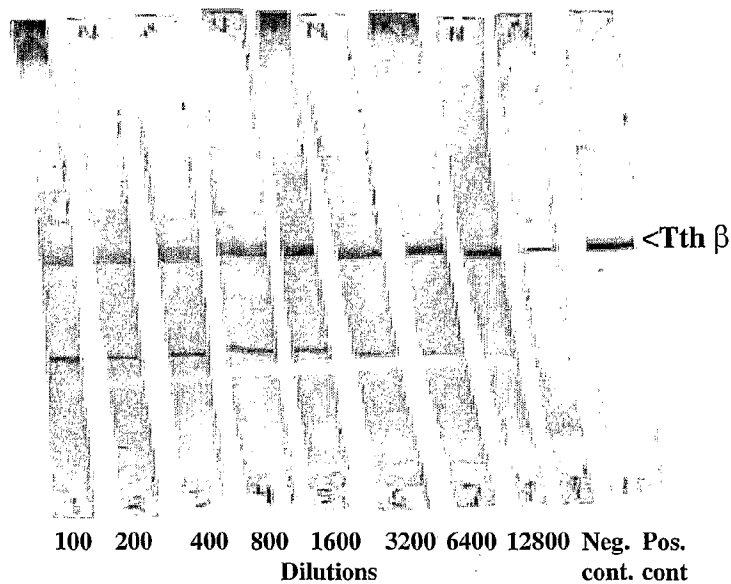
SDS-PAGE analysis of fractions eluting from a Sephacryl S-300 gel filtration column purification of *T. thermophilus*  $\beta$ .

36/63

**FIG. 36**

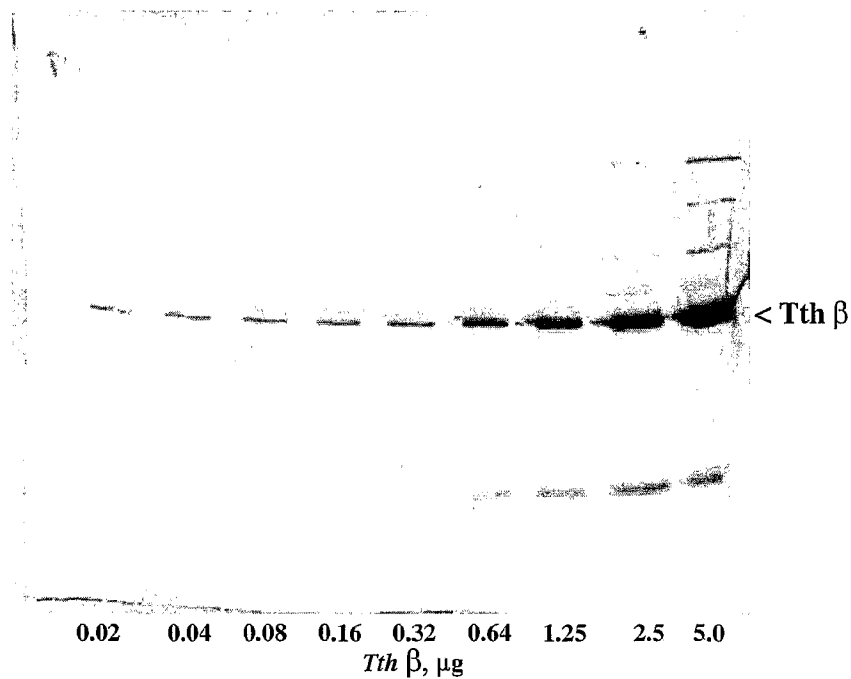
The pooled fractions of *T. thermophilus*  $\beta$  from the Sepharcry S-300 gel filtration column that was used in production of antibodies directed against  $\beta$ .

37/63

**Optimization of Tth Antiserum Concentration****FIG. 37**

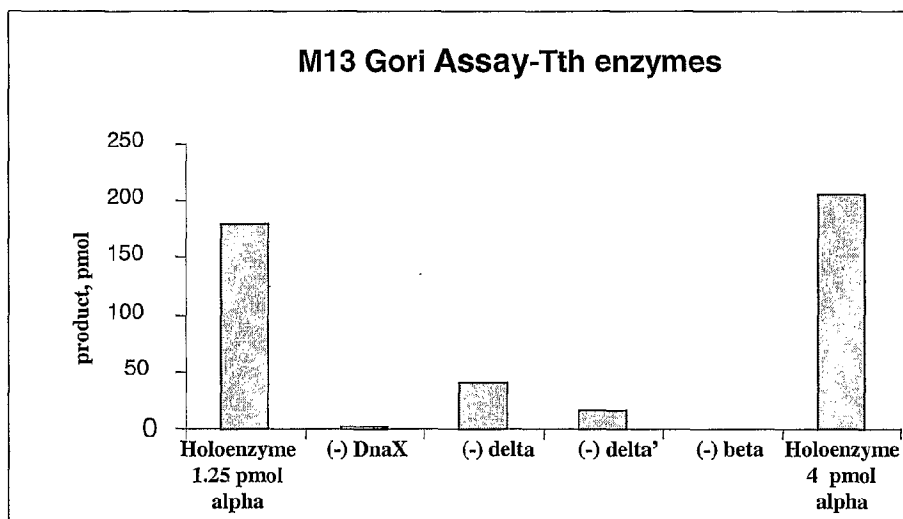
Western analysis of various antiserum dilutions for determination of dilutions to use in *T. thermophilus*  $\beta$  detection.

38/63

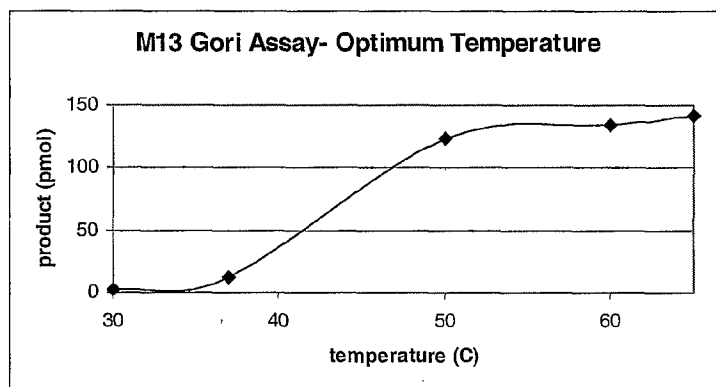
**Determination of the Limits of Tth  $\beta$  Detection****FIG. 38**

Western analysis of various *T. thermophilus*  $\beta$  dilutions for determination of the limit of  $\beta$  detection at antiserum dilution of 1:6400.

39/63

**FIG. 39****M13gori reconstitution of *T. thermophilus* Pol III subunits.**

40/63



**FIG. 40**  
Temperature dependence for a functional *T. thermophilus* holoenzyme in the reconstitution assay.

41/63

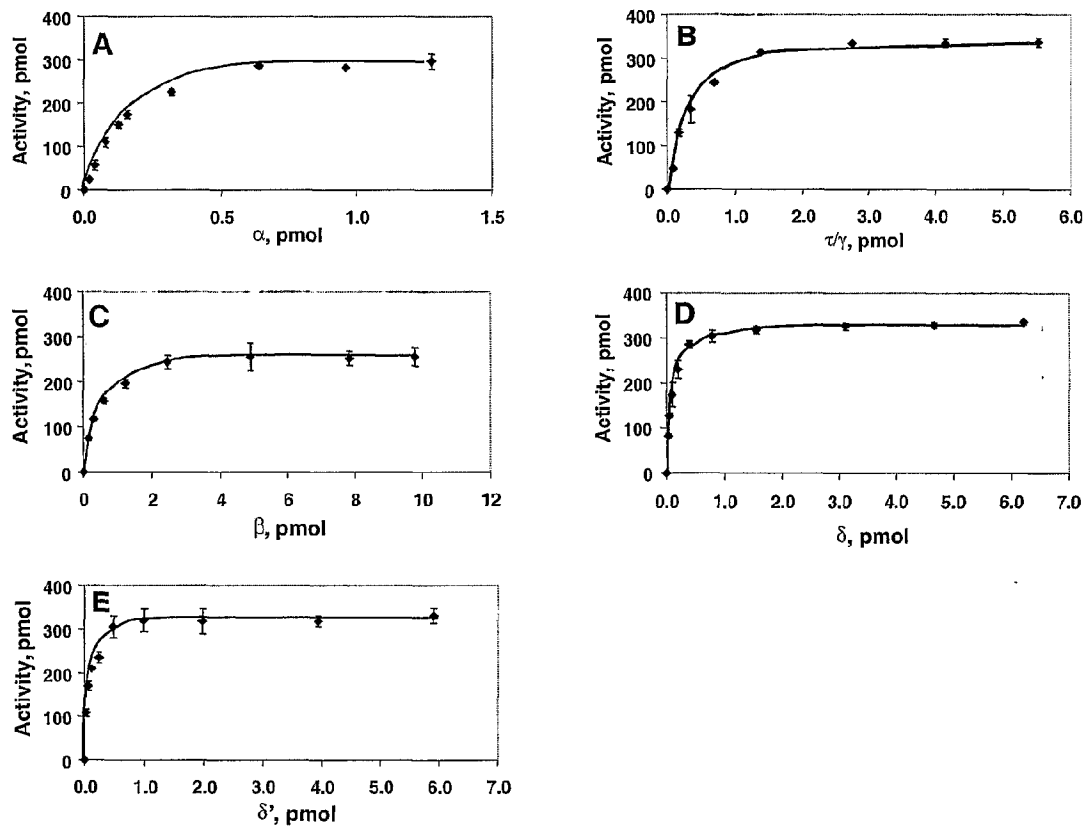


FIG. 41A-E

The reconstitution assay in which *T. thermophilus* A.  $\alpha$ , B.  $\tau/\gamma$ , C.  $\beta$ , D.  $\delta$ , and E.  $\delta'$  is/are titrated while the other subunits are held constant.

42/63

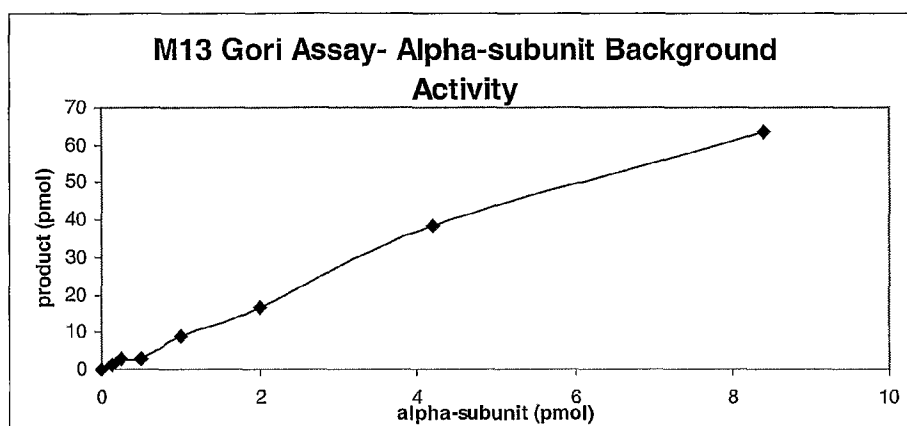
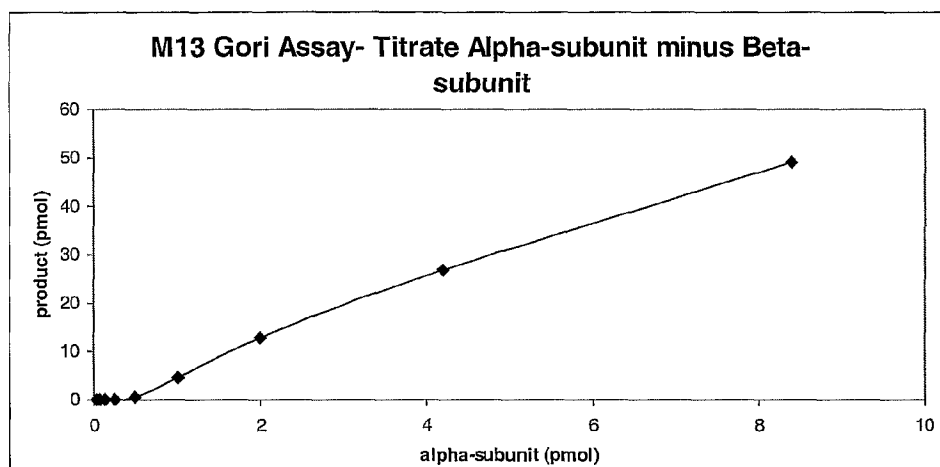


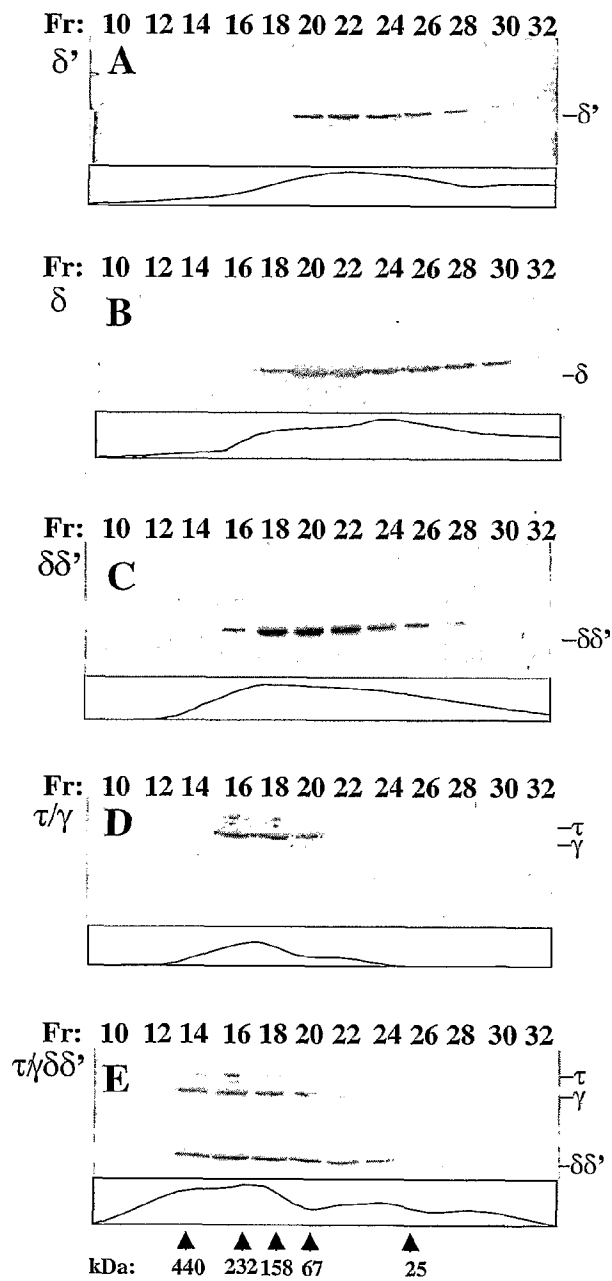
FIG. 42



43/63

**FIG. 43**

44/63



**FIG. 44A-E**  
Sephacryl S-200 gel filtration of subunits of the clamp loading complex showing protein-protein interactions.

45/63

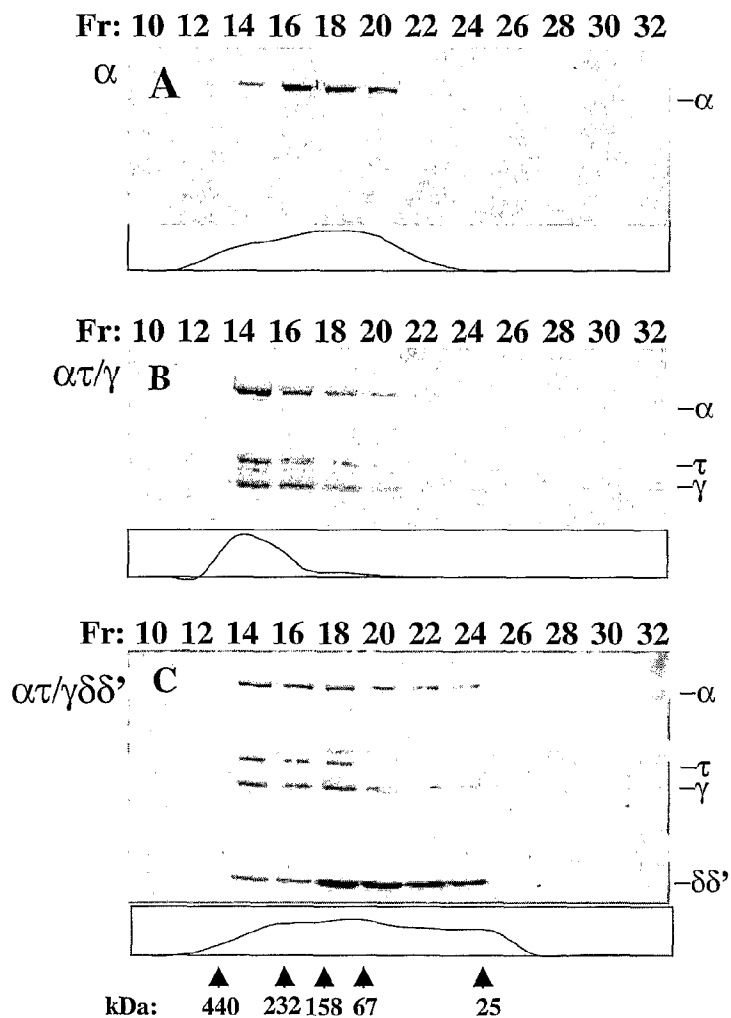


FIG. 45A-C

Sephacryl S-200 gel filtration of *T. thermophilus*  $\alpha$  with the subunits of the clamp loading complex showing protein-protein interactions.

46/63

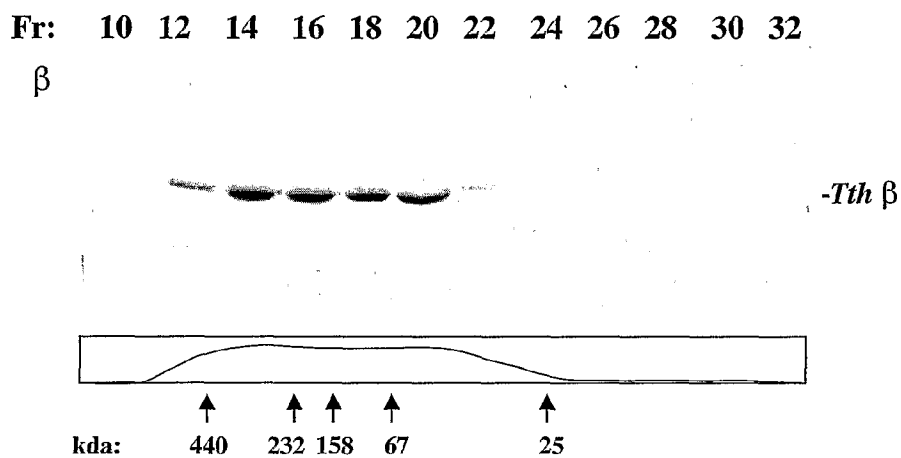


FIG. 46  
Sephacryl S-200 gel filtration of *T. thermophilus*  $\beta$ .

47/63

**SEQ ID NO:31:**

**at**ggctcgaggcctgaaccgcgttttctctcatcggcgccttcgccacccggccggacatg  
cgctacaccccgggcggtcgccattttgacctgacctcgccggtcaggacctgctc  
ctttccgataacgggggggagcgggaggtgtcctggtaccacgggtgaggctcttaggc  
cgccaggcggagatgtggggcgacctcttgaccaagggcagctcgtcttcgtggagggc  
cgctggagtagcgcagtgggaaaggagggggagaagcggagcagctccagatccgg  
gccgacttcttgacccctggacgaccgggggaaggagcggcgaggacagccggggc  
cagcccaggctccgcgcgcctgaaccaggctcttctctcatgggcaacctgacccgggac  
ccggaactccgctacacccccagggcaccgcggtggcccggtgggcctggcggtgaac  
gagcgcccgccagggggcgaggagcgcacccacttctgtggaggttcaggcctggcgcgac  
ctggcggtgagtgggcccgagctgaggaaggcgacggccttttctgtgatcggcagggtg  
gtgaacgactcctggaccagctccagcggcgagcggcgcttccagaccggtgtggaggcc  
ctcaggctggagcgcgccacccgtggacctgccaggccggcggaagcagggtcccgcgaa  
gtccagacgggtgggggtggacattgacgaaggcttggaagactttccgcggaggaggat  
ttgccgttt**tga**

**FIG. 47****DNA sequence of the gene encoding *T. thermophilus* SSB.**

48/63

SEQ ID NO:32

MARGLNRVFLIGALATRPDMRYTPAGLAILDLTLAGQDLLLLSDNGGEREVS  
SWYHRVRLLG  
RQAEMWGDLLDQGQLVFVEGRLEYRQWEREGEKRSELQIRADFLDPLDDRGKERAEDSRG  
QPRLRAALNQVFLMGNLTRDPELRYTPQGTAVARLGLAVNERRQGAEERTHFVEVQAWRD  
LAEWAAELRKGDGLFVIGRLVNDSWTSSSGERRFQTRVEALRLERPTRGPAQAGGSRSRE  
VQTGGVDIDEGLEDFPPEEDLPF

FIG. 48

49/63

		1		50
Aquifex SSB	(1)	---MLNKVFITGRLTGDEVIITVLPSCTPVVEFTLAYNRRYKQNN-GEFQ		
B. subtilus SSB	(1)	---MLNRRVVLVGRLTKDPELRYTPNGAAVATFLAVNRTFTNQS-GE-R		
E. coli SSB	(1)	MSARGINKVILVGRLGNDPEVRYLPNGCAVANLQVATSESWDRKQTGEMR		
H. influenzae SSB	(1)	--MAGINKVITVGHIGNDPEIRTMPNGDAVANISVATSESWNDRNTGERR		
TthSSB	(1)	-MARGLNRRVFLTGALATRPDMRYTEAGLATLDTLAGQDLLLSDNNGE-R		
Consensus	(1)	GLNKVILVGRLG DPEIRYIPNG AVA LTLA NESW N GE R		
		51		100
Aquifex SSB	(46)	ESHFFDVKAYGRMAEDWATRFSGVYLVEGRLSQEKWEK-EGKKFSKV		
B. subtilus SSB	(45)	E-ADFINCVTWROAENVANFLKKSLAGVDGRLQTRNYENQCCQRVEVT		
E. coli SSB	(51)	EQTWHRVVLFGLAEVAGEYLKKGCAQVYIEGQIRTRSDDD-NGITRYIT		
H. influenzae SSB	(49)	EVTWHRVVLFGRQAEICGEYLKKGCAQVYIEGRLKTRKQDQNGQDRYIT		
TthSSB	(49)	EVSQWYHRVRLGRQAEIWDLLDQGLVEVEGRLEYRQWER-EGEKISEL		
Consensus	(51)	E SEFHRVV YGRQAEI GEYLKKGSLVYVEGRL TR WE NG KRY T		
		101		150
Aquifex SSB	(95)	RIIA---ENVR-LINRPKGAELOAEE-----EEVFP		
B. subtilus SSB	(94)	EYQAESVQFLEPKNGGSGSGGYNEG-----N-----SGGGQYF		
E. coli SSB	(100)	ETLVKTTGTMQ-MLGSAPQQAQAQP-----KPQNGQ		
H. influenzae SSB	(99)	ETQG---DVMQ-MLGGR-NQNAAGGYG-----N-DMGGA		
TthSSB	(98)	QTRADFLLDPLD-DRCKERAEDSRGQPRRLRAALNQVFLMGNLTRDPELRYT		
Consensus	(101)	EI A D L MLG G A A E		
		151		200
Aquifex SSB	(122)	PIEEEIEKL-----CKEEKPTDEEDELFF-----		
B. subtilus SSB	(128)	GGGQNDNPFQGNQNN--QRRNQGNSEND-DPFANDGKP-IDISDDDLFF-		
E. coli SSB	(132)	PQSADATKKKGAKTK---CRERKAAQPEPQPTTEGED--YGFSDDLFF-		
H. influenzae SSB	(126)	PQSSYQAR---QTN--CNSYQSSRPAPQQAEPQAEPPMDGFDDDLFF-		
TthSSB	(147)	PQGTAVARLGLAVNERROGAEERTHIVVQAWRDLAETWAAFLRKGDLFFV		
Consensus	(151)	PQ K G N GRE SF D Q P AE D DDIPF		
		201		250
Aquifex SSB	(148)	-----		
B. subtilus SSB	(173)	-----		
E. coli SSB	(176)	-----		
H. influenzae SSB	(169)	-----		
TthSSB	(197)	IGRLVNDSWTSSSGERRFQTRVEALRLRPTRGPAQAGGSRSEVQTGGV		
Consensus	(201)			
		251		267
Aquifex SSB	(148)	-----		
B. subtilus SSB	(173)	-----		
E. coli SSB	(176)	-----		
H. influenzae SSB	(169)	-----		
TthSSB	(247)	DIDEGLEDFPPEEDLFF		
Consensus	(251)			

FIG. 49

Sequence alignment of *T. thermophilus* SSB compared with SSB amino acid sequences from *Aquifex*, *B. subtilus*, *E. coli* and *H. influenzae*.

50/63

```

1                               50
C-termTthSSB (1) ---ALNQLVFLMGNLITRDPELRYTPQETAVARLGLAVNERRQ--GAEER--
N-termTthSSB (1) MARGLNRFVFLIGALATRPDMRYTPAGLAALDLITLAGQDLLLSDNIGGEREV
Consensus (1) ALN VFLIG L PDLRYTP G AI L LA ND A ER
51                               100
C-termTthSSB (44) --TFVFEVQAWRLABTAABTRKGDGLFVIGRLVNDSTSSSGERRFQTR
N-termTthSSB (51) SWYERVRRLG-RQAEMTGDLIDQGLVFEGRLEYRQERE-GEKRSELQ
Consensus (51) H V L A R WA L G LFV GRL W GEKR
101                               147
C-termTthSSB (92) VEALRLERP-TRGPAQAGGSRSREVQTGGVDIDEGLEDFPPEEDLFF
N-termTthSSB (99) ITRAFELDPLDDRGKERFEDSRGQPRLR-----
Consensus (101) I A LD RG A SR A

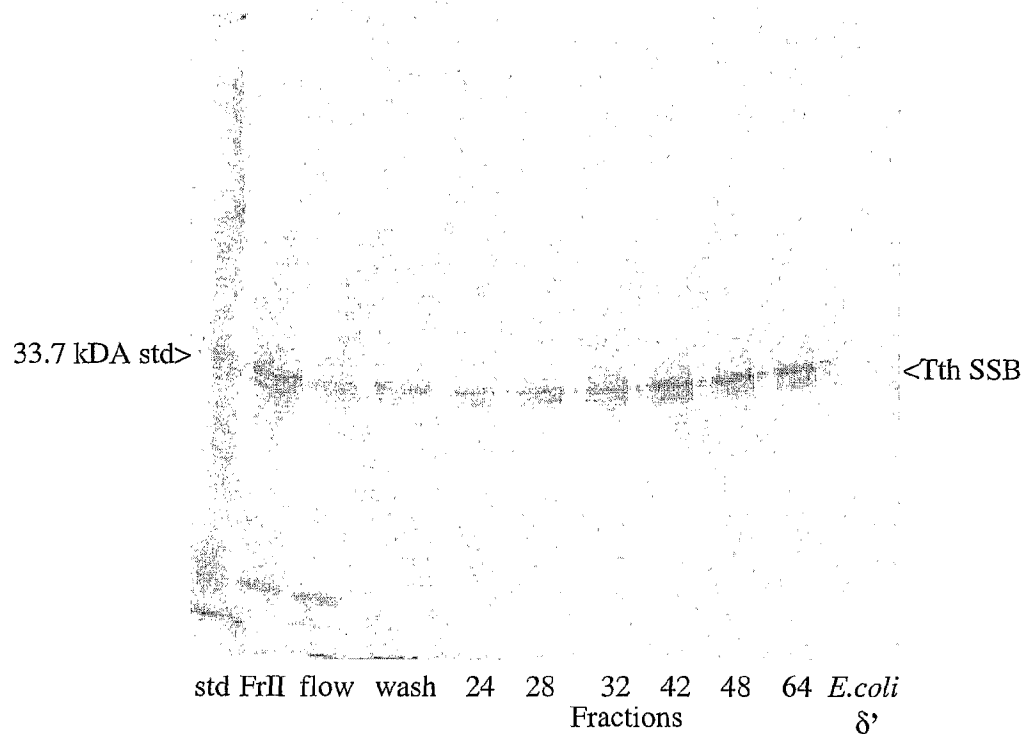
```

FIG. 50

Sequence alignment of the N-terminal region of *T. thermophilus* SSB with the C-terminal region of *T. thermophilus* SSB.



51/63

**Biotin Blot Analysis of Ni-NTA Column Profile- *Tth* SSB****FIG. 51**

**Biotin blot analysis of relevant fractions from the  $\text{Ni}^{++}$ -NTA column purification of *T. thermophilus* SSB.**

52/63

**SEQ ID NO:36**

**gtg**cctgcaggctctggaggacgtcaccctggaccagctcctttacaccgcctttgacctg  
gagaccacggggcctggacccggagcaagatgccgtggtggccctcgccgggggtccatatac  
ctgggtcgaagggctcttgccgcaggaggtgttcgaggccctggtgaacccggggcgcccc  
atctcccccgccggccacggcgggtccacggcctcacggcggagatgctccgggacaagcct  
cccctagaggcgggtcctccccgccttccgcgccttcgtccaggacacgggtgctggtggcc  
cacaacggggcctttgacctggcctttctgcgcggggcgggcctggaccagccccccctc  
ctggacacctcctcctcgcccagctcctcttccccgacctcaaggactaccgcctcgag  
gccctggcccaccgcttcggcgtccccgccaccggggcggcacaccgccttgggcgatgcg  
ctgatgacggcggaggctcttcgtgaggatgcagccctcctctttgaacgggggcttagg  
cggctctgggacgtggtggaggcctgccgcgcctcccccttgggcccggtcaggtact**ga**

**FIG. 52**

53/63

**SEQ ID NO:37**

VPAGLEDVTLDQLLYTAFDLETTGLDPEQDAVVALAGVHILGRRVLRQEVFEALVNPGRP  
ISPAATAVHGLTAEMLRDKPPEAVLPAFRAFVQDTVLAHNGAFDLAFLRRAGLDQPPL  
LDTLLLAQLLFPDLKDYRLEALAHRFVGPATGRHTALGDALMTAEVFVRMQPLLEFERGLR  
RLWDVVEACRRLPLARLRY

**FIG. 53**

54/63

SEQ ID NO:67

**gtg**catagcgacgccctcctagccccctcaacgaggcccagcgccaggcggtcctccac  
tttgagggggccccgacctggtggtggcgggggcgaggcggaagaccgcaccgtggtc  
caccgggtggcctacctcgctcgcccgccgggggtcttccccctcgagagatcctggccgtc  
accttaccacaagaaggccgaggagatgcgggagcgccctccgggggtggtcccgggg  
gcgggggagggtctggtctccaccttccacgcccgccttgaggatcctccgcgtctac  
ggggagcggggtggcctcaggccccggtttgtggtctacgacgaggacgaccagaccgcc  
ctcctcaaggagggtcctgaaggagctcgccctctcgccccggccccggccccatcaaggcc  
cttttggaccggggaagaaccggggcggtgggccttaaggccctcctcggcgagcttccc  
gagtactacgcccgggttttcccggggaaggcttggggacgctcctggtgcgctaccaggag  
gcccttaaggcccagggggccttggacttcggggacatcctcctctacgcccgtgaggctt  
ttagaggaggacgaggagggtcctcaggctcgctgcgaagcgggcccgcttcacccacgtg  
gacgagtaccaggacacgagccccgtccagtagcgccttaccgggttctctcgccggggag  
gaggccaacctcatggcggtggcgacccccgaccaggggatctactccttccggggcgcg  
gacatcaagaacatcctggacttaccggggactacccccgaggccccgggtctaccgcctg  
gaggagaactaccgctccaccgaggccatcctccgcttcgccaacgccgtgatcgtaag  
aacgccttccgctggagaaggccctgcgcccgggtgaagcggggcggggagcccgtgcgc  
ctctaccggggcgaggacgcCcgggaggaggccccgcttcgtggccgaggagatcgccccg  
ctcgccccccccctgggaccggtacgcccgtcctctaccgcaccaacgccccaaagccgcctt  
ctggaacaggcggttggcggggcgggggatccccgcgcgggtggtggggcgcggtgggggtt  
tttgaaagggccgaggtcaaggacctcctggcctacgccccgctcgcctcaacccccctg  
gacgccgtgagcctgaagcggtcctgaacacccccctcggggcacgccccggccacc  
tgggccagggtgcagctcctcgcccaggagaaggggcttccctcctgggaggccctgaag  
gaggcgccaggaccttcccccgccgagccctgaggcacttcgtggccctggtggag  
gagcttcaggacctggtcttcggccccgcgaggcccttcttccgccacctcctcgaggcc  
accgactacccccgcctacctccgggaggcctacccccaggacgcccaggaccgcctggag  
aacgtggaggagctcctcaggggcgcccaaggaggcgaggacctgcaggacttcttgag  
cggtggccctcaccgccaaggcgaggagccccgcccaggcgagggggagggtcgcctc  
atgacctgcacaacgccaaggggtggagtccccgtggtcttccctcgtaggggtggag  
gaggggtctctgccccaccgcaactccgtgagcaccctcgaggccctggaggaggagcgc  
cgctctctctacgtgggcatcaccgggcccaggagaggctctacctctccacgcccag  
gagcgggagggtctacggcaggcgggagccccgcccggcgagccgcttccctggaggagggtg  
gaggaggggctttacgagggtgtaacgaccttaccggcgccccgcccctccccgccccccat  
cgcccagggcccggggcttccggggcggggagcggggtggtccacccccgcttcggcccc  
ggcaccgtggtggcgggccagggggacgagggtcacgggtccactttgaggggtttgggctc  
aagcgcccttccctcaagtacgcgagcttaagccggcat**ga**

FIG. 54

55/63

SEQ ID NO:68

VHSDALLAPLNEAQRQAVLHFEGPALVVAGAGSGKTRTVVHRVAYLVARRGVF  
PSEILAVTFTNKAAEEMRERLRGLVPGAGEVWVSTFHAAALRILRVYGERVGLR  
PGFVVYDEDDQTALLKEVLKELALSARPGPIKALLDRAKNRGVGLKALLGELPE  
YYAGLSRGRLGDVLVRYQEALKAQGALDFGDILLYALRLLEEDEEVLRLVRKRA  
RFIHVDEYQDTSPVQYRFTRLLAGEEANLMAVGDPDQGIYSFRAADIKNILDFT  
DYPEARVYRLEENYRSTEAILRFANAVIVKNALRLEKALRPVKRGGEVRLYRA  
EDAREEARFVAEEIARLGPPWDYAVLYRTNAQSRILLEQALAGRGIPARVVGGV  
GFFERADEVKDLLAYARLALNPLDAVSLKRVLNTPPRGIGPATWARVQLLAQEK  
LPPWEALKEAARTFPRAEPLRHFVALVEELQDLVFGPAEAFRHLLEATDYPAYL  
REAYPEDAEDRLENVEELLRAAKEAEDLQDFLDRVALTAKAEPAEAEGRVAL  
MTLHNAKGLEFPVVFLVGVEEGLLPHRNSVSTLEGLEEEERLFYVGITRAQERLY  
LSHAEEREVYGRREPARPSRFLEEVEEGLYEVDYPYRRPPSPPPHRPRPGA  
FRGGERVVHPRFGPGTVVAAQGDEVTVHFEGFGLKRLSLKYAELKPA

Fig. 55

56/63

SEQ ID NO:71

atggacgcggggccaggcggtggagctgatcaagtcccgcctctccttgcgggaggtggtc  
tcccgctacgtggccctgaagccggcgggccgcggccgctggaagggcctctgcccttc  
caccaggagaagacccccctctttctacgtggacgaggagaagggcctcttctactgcttc  
gggtgcaaggccgggggacgacctcttcgccttcgtccaaagggcggaggggctggacttc  
cccgaggccctggaaaggctcgcggaggaggcgggggtggagcttccccggcggaaggcc  
cggaaaggcgccgggagctcctcgaggtcctggccctggcccagacctacttccctggag  
cacctccacgcccacccccaggccctggcctacctgaggaagcggggcctcacggaggag  
agcgtggcccgcttcggcctgggctacgccccgcccagggggacgggctcgtggccttc  
ctcgccccgcacggggtggccccggaggaggcgctgcgggccccgggtgctggcggagcgg  
caggggctcgttggaacgcctccgccaccgcacaccttccccatcaaggacgccttc  
gggcgcgtcgtggccttcaccgggaggggcctgggggaggacggggcccaagtacctgaac  
acccccgaaacccccctttccgcaagcaggaggctcctcttcgcctacccccaggcccg  
ccgccttgaggaggggcgggccatcgctgggtggaggggctctttgacgccatcgccctc  
caccagctgggcttcccgagacggtggcgtgctgggctcgggcctctccgaggacag  
gccctcctcctcaagaaggcgggggtcctggaggtctacctggcctttgacgccgacgag  
gccgggcagaaggccaccttgacagacctgaacctggagctcgcctccagggttccctcttc  
tacgcgtccgcctccccgccaaggacccgggggagctcctcctccacccccagggaagg  
gccctcttccagaaggccctggaggaggccctccccgagggtggccttccgctttgaggag  
gcgagccgggggctggacctctcccgccccgagcacaagcggaaggctcctcgaggccctc  
acccccaggatgctcaccgccgagccctttgatcccggtggccgagcggctcaaggccctg  
gtggtggagcgcctgggccttcccccaagagcctcgaggactacctggcgagccttagg  
acccgggggcgggcccgccctccccacgcgcccccacccctcaccctccggcaacaagacc  
ctccttctggagctggacgccatcgccctcctcctctccgccccggaggagcgttccctg  
gagcttgtggactacgtggagaccaggtctggcccccgagggttccctcctcggggag  
ttcctggccctggccccgaaggagccccggcgggaccacctccgcccacacctaaagccag  
cgggaggaaggaggaaggctctttgagcgctgctcctcgcgccccgggggaggatccc  
aggctccaggagaagctggaccacacctggccccgctcggggaagcctacctccaggag  
cggctcgccaaggctcaaggcgccctcgcccaaaacccccgacccccccacctggagcgc  
ctccttaaggagtaccaggagatccgggtggccatagaggcgagcgcgcctctacaag  
cggcgccccctccttcgggctggtccacctag

Fig. 56

57/63

## SEQ ID NO:72

MDAGQAVELIKSRLSLREVVSRYVALKPAGRGRWKGLCPFHQEKTPSFYVDEEKGLFYCF  
GCKAGGDLFAFVQRAEGLDFPEALERLAEAEAGVELPRRKAPERRELLLEVLALAQTYFLE  
HLHAHPEALAYLRKRGLTEESVARFGLGYAPPKGDGLVAFRLARHGVAPEEGVRAGVLAER  
QGRFVDRLRHRITFPKDAFGRVVAFTGRALGEDGPKYLNTPETPLFRKQEVLFAYPEAR  
PALREGRAIVVEGLFDAIALHQLGFPETVAVLGSGLSEGQALLKKAGVLEVYLAFDADE  
AGQKATLQSLNLELAPRFLFYAVRLPAKDPGELLHPEGRALFQKALEEALPEVAFRFEE  
ASRGLDLSRPEHKRKVLEALTTPRMLTPEPFDPAERLKALVVERLGLSPKSLEDYLASLR  
TRGRPAPPPPPPLTPGNKTLLELDAIALLLSAPEERFLELVDYVETQVWPPEGSFLGE  
FLALARKEPRRDHLRRTLSQREEGGRLFERLLLAPRGEDPRLQEKLDHTLARLREAYLQE  
RLAKVKAALAQNPDPTLERLLKEYQEIRVAIEAERRLYKRRPPPSGWST

Fig. 57

58/63

SEQ ID NO:75

gtgcggggtgcttcaggtggcccttccccctgccgcttccgcccattgagctacctccctccc  
ttgggggcaggagggagaggagggccttggggcgggcggtggccgttcccttttcgggggggag  
gtccaggtgggggtggtggtgggggaagggggcgggccctccctgaacctccgccacgcc  
atcgccctacctggacccccgccccctacctgcgcccggaggagatcctcttttttgggaagag  
gcggccccgtaccttttcgcccccttggggccaggtcctggcggaacttccctcccccccttt  
cccccttgcgccaccgggtccgcctctacccggggggcggaaccgaaggtcctgcccccg  
gggctcggggccttgggtggactggcgggaggcccggggctttgaccccaagcttttggac  
ctcctgcgggaggcggggatgctggaggaggagctcgccttccgggaggcgcggggggtg  
ctgggtgcccctgaagcccgcccaccccgatccccagctggaccgcgtccttcaggtcctg  
cgggagctgggctttgcccgaagccaggcgggccttggcccggggcgcggggggtgggggtg  
ggccgggtgcgcgcctcgtccaggaggggtacatcggcacccgcgtcccccgaggaggcc  
gccccgcccccgggcggaagggtggacgtggcgccccctccacctccccgagaggcccgag  
cgggtcaacggcggggaggtttctggaacgcgttcgggtgctcaaggggcttttgggggag  
ggggaccacctgggtcctcttccccgaggtgagcctcttggagcgggtttctcgccccacttc  
ccccggggccacgcctaccacggggggctttccggccccggtccgggagcgggtttttccgg  
aggccgcgcggcggtggtcttgcgcacctacggcgggctcctcctcccccttccccccgc  
tctttggtggtggtggaggaggggagcgagagctacaagcttccctcggggagcggggcc  
ttcgtccccccgcttgcggagcttagggcccggtcctcctgggggtgccccctacctacctc  
tccctggtgcccgcggtggaggttttggagcgaaaaggcttcgccttgcctgcccgtgcccaag  
ccccgccttctcctcttggacctccggcggggagcggggcttcccggtcacggggcggggc  
ctcgccctcctccgccaggtggaggagcggggcgggcaggccgtggtcctctccgcccgc  
aaggggtacagcgccctcctcctctgccaggactgcggcttccggcccattgtgccccgac  
tgcccttggccctgcggtaccacccgggaggggaagggggcgctcgtctgccaccagtg  
ggccaccgcgaggaccgccctcctctgcccccggtgcggctccccctcctcgcccc  
aaggggcccgggggtggactggatccgggaggccctggcgaggaggtttcccttcccgctc  
taccgctacgccggcgacgggaaggacgacctccccccctcctcgaggggccggccgggg  
gtggtggtggggaccacggccctcctcagggggcctaggcttcccgacctcgccctcgtc  
ctcctccctttggcggaaggcttctccttggaagtcggacttccgggcggcgaggcgggtac  
caccgccttctctgggcccctcacggagctcaggccccgggcggaggccccctcctcgtcctc  
cagaccttccccccgagcaccccggtgcacccggccccctcgaggcgggggaggtggaggcc  
tacctgtggcaggagaaggccctgcgggaggccctcaactaccgcgcgggtgcgcatg  
gtaaagctggagggtgcgccaccgaaaggaagagcggggcccggaagggccttcgcctc  
ctggaggccttgcgggcccaggcgaggagggggaggtcctgggccccgcccccgctccc  
gtgccccgggtgaaggggcattacgtcttccacctcctcctccgggggagcacggagcgg  
ctcgccccgcctcctcgccctcctggaccggcggcagttcaggctggacccccgaccccttc  
cacttcgtggggcttttggaggactag

Fig. 58



59/63

SEQ ID NO:76

VRVLQVALPLPLPMSYLPPLGQEGEEALGRRVAVPFRGEVQVGVVVGEGGRPSLNLRHA  
IAYLDPAPYLRPEEILFLEEAARYLFAPLGQVLADFLPPFPPLRHRVRLYPGADPKVLPP  
GLGALVDWREARGFDPKLLDLLREAGMLEEELAFREARGVLVPLKPAHPDPQLDRVLQVL  
RELGFAESQAALARAAGVGVRRLVQEGYIGTASPEEAAPPPADGVDVAPLHLPERPE  
RVNGGRFLERVRLKGLLGEGDHLVLFPEVSLLERFLAHFPGATPYHGGLSGPVRERFFR  
RPRGVVFATYGGLLLPFTPRSLVVVEEGSESYKLPSGSRAFVPPPLAELRARLLGVPLTYL  
SLVPAVEVLERKGFALPVPKPRLLLLDLRRERGFVPTGRALALLRQVEERGRQAVVLSAR  
KGYSALLLCQDCGFRPMCPDCALPLRYHREGKGALVCHQCGHREDPPLLCPRCGSPLLAP  
KPGVDWIREALAERLSLPVYRYAGDGKDDLTPLLEGRPGVVVGTTALLRGPRLPDLALV  
LLPLADGFLLESDFRAAERYHRLWALTELRPGRRPLVLQTFTPEHPVHRALEAGEVEA  
YLWQEKALREALNYPPVRMVKLEVRHRKEERAREKAFALLEALRAEAEEGEVLGPAPAP  
VPRVKGHYVFHLLLRGSTERLARLLGLLDRRQFRLDPDPFHFVGLLED

Fig. 59

60/63

SEQ ID NO: 81

gtggagcgggtggtgcggcccccttctggacgggaggttcctcctggaggaggggg  
ctttgggagtggtgcgtacccctttccctggagggggagtgtggtcctggacct  
gagaccacggggcttgccccgggcctggacgaggtgattgaggtgggcctcctc  
gaggggggggaggcgccctcccccttccagagcctcgtccgcccttcccggccgcg  
ttcgtggagcgcctcacccggcatccccgggaggccctggaggaggccccctcc  
gaggttctggagaaggcctacccccctcctcgccgacgccaccttggtgatccac  
gcctttgacctgggcttcctccgcccggccctggagggcctgggctaccgcctg  
cccgagggtgactccctgcgcttggccagacggggcttaccaggccttaggcgc  
ctggacgccctctccgaggtcctggagcttccccgaaggacctgccaccggggcc  
gacgtggagcgcaccctcgccgtggtgcacgaggtgtactatatgcttacgtcc  
ccccgcacgctttgggaactcgggaggtag

Fig. 60

61/63

SEQ ID NO: 82

VERVVRPLLDGRFLLEEGVGLWEWRYPPFLEGEAVVLDLETTGLAPGLDEVIE  
VGLLRLEGGRRLPFQSLVRPSRPPSPFVERLTGIPREALEEAPSLEEVLEKAYPLLA  
DATLVIHNAAF DLGFLRPALEGLGYRLENPVVDSLRLARRGLPGLRRYGLDALS  
EVLELPRRTCHRALEDVERTLAVVHEVYYMLTSGRPRTLWELGR

**Fig. 61**

62/63

SEQ ID NO: 22

5' UTR

gtgaccggggccccctcgaggtcgacggtatcgataagcttgatatcgaaaaagagagtcgtaatagccgt  
taaaggaggcgtcgggca

ATGAACATAACGGTTCCCAAGAACTCCTCTCGGACCAGCTTTCCTCTCGGAGCGCATCGTCCCCCTCTAG  
AAGCGCCAACCCCTCTACACCTACCTGGGGCTTTACGCCGAGGAAGGGGCCTTG  
ATCCTCTTCGGGACCAACGGGGAGGTGGACCTCGAGGTCCGCC'TCCCCGCCGAGGCCCAAAGC  
CTTCCCCGGGTGCTCGTCCCCGCCAGCCCTTCTTCCAGCTGGTGCGGAGCCTTCCTGGGGAC  
CTCGTGGCCCTCGGCCTCGCCTCGGAGCCGGGCCAGGGGGGCGAGCTGGAGCTCTCCTCCGGG  
CGCTTCCGCACCCGGCTCAGCCTGGCCCTGCCGAGGGCTACCCCGAGCTTCTGGTGCCCGAG  
GGGAGGACAAGGGGGCTTCCCCCTGCCGACGCGGATGCCCTCCGGGGAGCTCGTCAAGGCC  
TTGACCCACGTGCGCTACGCCCGGAGCAACGAGGAGTACCGGGCCATCTTCCGCGGGGTGCAG  
CTGGAGTTCTCCCCCAGGGCTTCCGGGCGGTGGCCTCCGACGGGTACCGCCTCGCCCTCTACGACCTGCC  
CCTGCCCCAAGGGTTCCAGGCCAAGGCCGTGGTCCCCGCCCGGAGCGTGACGAGATGGTGCGGGTCTCTGA  
AGGGGGCGGACGGGGCCGAGGCCGACCTCGCCCTGGGCGAGGGGGTGTGGCCCTGGCCCTCGAGGGCGGA  
AGCGGGGTCCGGATGGCCCTCCGCCCTATGGAAGGGGAGTTCCCCGACTACCAGAGGGTCATCCCCAGGA  
GTTTCGCCCTCAGGGTCCAGGTGGAGGGGGAGGCCCTCAGGGAGGCGGTGCGCCGGGTGAGCGTCTCTCCG  
ACCGGCAGAACCACCGGGTGGACCTCCTTTTGGAGGAGGGCCGGATCCTCCTTTCCGCCGAGGGGGACTAC  
GGCAAGGGGCAGGAGGAGGTGCCCCGCCAGGTGGAGGGGCCGGCATGGCCGTGGCCTACAACGCCCGCTA  
CCTCCTCGAGGCCCTCGCCCCCGTGGGGGACCGGGGCCACCTGGGCATCTCCGGGCCACGAGTCCGAGCC  
TCATCTGGGGGACGGGGAGGGGTACCGGGCGGTGGTGGTGCCCTCAGGGTCTAG

3' UTR

ggggtagtatggggcgggggagcgtgaagcgcttccacaagggaggttgggcatgaccaccatcgctggcg  
tccggggcacgcgaggttttggattccaggggcttccacggttagaggcggaggtggagctggaaggcggg  
gccaggggcccggccatggtgccctccggggcctccaccggaacccacgaggccctggagctcagggacgg  
cggcaagcgctacctgggcaagggggtgcgccggggggtggagaacgtcaacgagcgcatcgccccgagc  
tcgtcggcatggacgccctggaccaggaaggggtggaccggggccatgctggagctggacggcacccccac  
aaggccaacctgggagcgaacgcctcctcgcggtctccttggccgtggcccgggcccggcgagggccct  
gggectgcccctttaccgctacctggggcggggtccagggggtcacctgcccgtgcccctcatgaacgtca  
tcaacggggggaagcacgcgcgacaacgggtggacttccaggagtcatgctggtgcccgggggcgggga  
agcttcgccgaggccttgaggatcggggcccagggtcttccacacctcaaggccgtcctcaaggagaaggg  
ctacagcaccaacgtgggggacgagggaggttcgcccccgacctcaggagcaacgaggaggcgggtggagc  
ttttgctcctcgccattgagcgggcccgggtacaccccggggccaggaggtctccttggccctggaccggcc  
acgagcgagctttaccgggacgggaagtaccacctggaaggggagggcaaggctcctcctcggaggagat  
gggtggccttctgggaggcctgggtggagaagtaccccatccgctccattgaggacggccttgccgaggagc  
actgggaggggtggcggtctctcaccgagcgctgggggggaaggtccagctcgtgggggacgacctcttc  
gtcaccaacccgaaaggctccgggcccgggattgagcggggggtggccaacgccatcctggtcaagggtgaa  
ccagatcgggacctctcggaacacctcgaggccatccgcttgcccagcgctcgggggtacagggcgggtga  
tcgagaattc

Fig. 62

63/63

SEQ ID NO: 23

amino acid sequence

MNITVPKKLLSDQLSLLERIVPSRSANPLYTYLGLYAEEGALILFGTNGEVDLEVRLPA  
EAQSLPRVLVPAQPFFQLVRS LPGDLVALGLASEPGQGQLELSSGRFRTL SLAPAEG  
YPELLVPEGEDKGAFPLRTRMP SGELVKALTHVRYAASNEEYRAIFRGVQLEFSPQGFR  
AVASDGYRLALYDLPLPQGFQAKAVVPARSVDEMVRVLKGADGAEDLALGEGVLALAL  
EGGSGVRMALRLMEGEFPDYQRVIPQEFALRVQVEGEALREAVRRVSVLSDRQNHRVDL  
LLEEGRILLSAEGDYGKGQEEVPAQVEGPGMAVAYNARYLLEALAPVGDRAHLGISGPT  
SPSLIWGDGEGYRAVVVPLRV

Fig. 63